PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
X-11650		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/15018	08/06/2000	15/07/1999
International Patent Classification (IPC) or national classification and IPC C07K7/06		
Applicant ELI LILLY AND COMPANY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/01/2001	13.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Groenendijk, M Telephone No. +31 70 340 3715

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/15018

I. Basis of th report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21	as originally filed				
Claims, No.:					
4,5 (part),11 (part), 12	as originally filed				
1-3,5 (part),6-10, 11 (part)	as received on	12/07/2001	with letter of	12/07/2001	

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-12
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-7
	No:	Claims 8-12
Industrial applicability (IA)	Yes:	Claims 1-12
	No:	Claims

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following document/s/:

D1:FEBS Letters,, 355(1994), 96-100
D2:Agric.Biol.Chem., 53(2),1989, 497-504
D3:EP-A-0460882

I.NOVELTY

In view of the available prior art the claims 1-12 are considered to be novel under Art.33(2) PCT.

II.INVENTIVE STEP

1)The closest prior art is considered to be D1, disclosing peptides of the so-called pseudomycin and syringomycin groups and their use as antibiotics.
2)The present application differs from said prior art essentially therein that said compounds are enzymatically deacylated in order to provide the cyclic nucleus which can be used to synthesize sidechain analogs.
3)The problem to be solved may therefore be considered to be the provision of intermediate products of pseudomycin and syringomycin antibiotics as means for preparing sidechain analogs.
4)The solution is a process as defined in claim 1 wherein the sidechain is enzymatically removed by an ECB or polymixin deacylase.
5)It is true that ECB and polymixin deacylase were already known to be able to hydrolyse, in addition to echinocandin and polymixin type of compounds, also related lipopeptides or fatty acid-amino acid adducts (e.g., see D2 and D3).
However the use of said enzymes for the present deacylation has not been indicated or suggested and due to the different structures of the present compounds it could not be expected that the present compounds would actually be properly deacylated, which view is supported by the fact that only selected enzymes appear to be suitable (see description page 9, lines 7-8).
5)Consequently an inventive step can be acknowledged for a process (and the intermediate compounds) as defined in claim 1 as far as it solves the problem posed.

However, as indicated in the previous paragraph, it cannot be predicted whether certain enzymes actually can hydrolyse the present compounds. At present only Pseudomycin A has been demonstrated to be hydrolysed. In order to acknowledge an inventive step to a process for deacylating Syringomycins, which compounds have a different surrounding of the site of hydrolysation, and the obtained intermediate products, that is, the subject-matter of the claims 8-12, additional experimental data should have been filed. In the absence of said data the subject-matter of the claims 8-12 is considered not to solve the problem posed and hence no inventive step can be acknowledged for said claims under Art.33(3) PCT.

Re Item VII

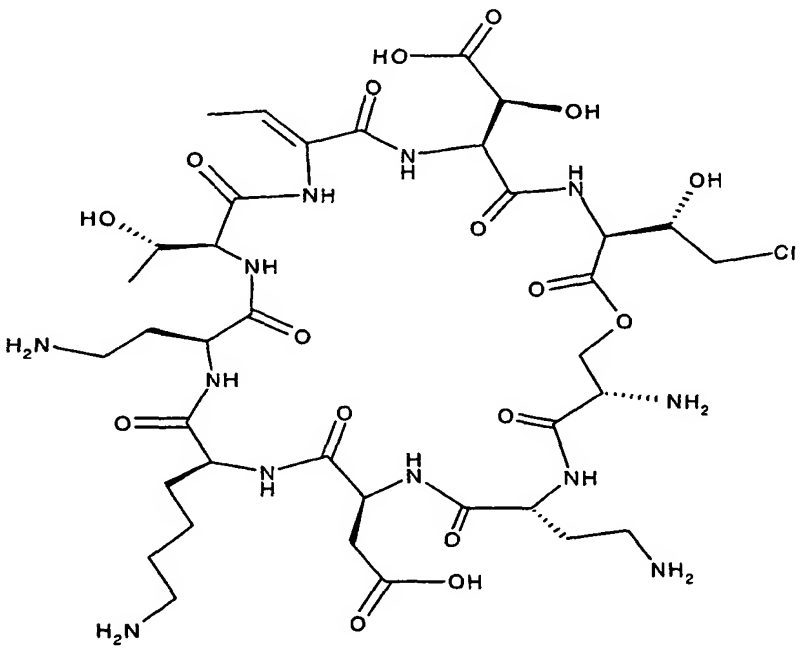
Certain defects in the international application

In several positions in the description the application contains the expression "incorporated by reference". However the application should, regarding the essential features of the invention, be self-contained, that is, capable of being understood without reference to any other document. Consequently said expression should have been deleted from the description and, if necessary, said subject-matter by reference should expressly have been incorporated into the description, subject to the restrictions under the Articles 19(2) and 34(2)(b) PCT (see PCT Guidelines CII, 4.17-4.18).

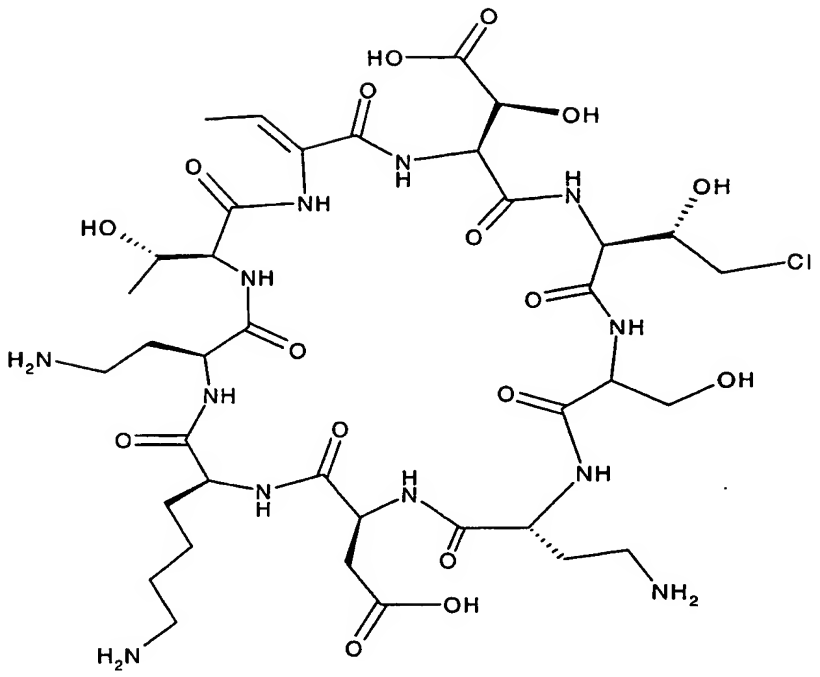
WE CLAIM:

1. A process for deacylating an N-acyl side-chain of a pseudomycin natural product comprising the step of reacting a pseudomycin natural product with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce a pseudomycin nucleus.

2. The process of Claim 1 wherein said pseudomycin nucleus is represented by either structure I or II



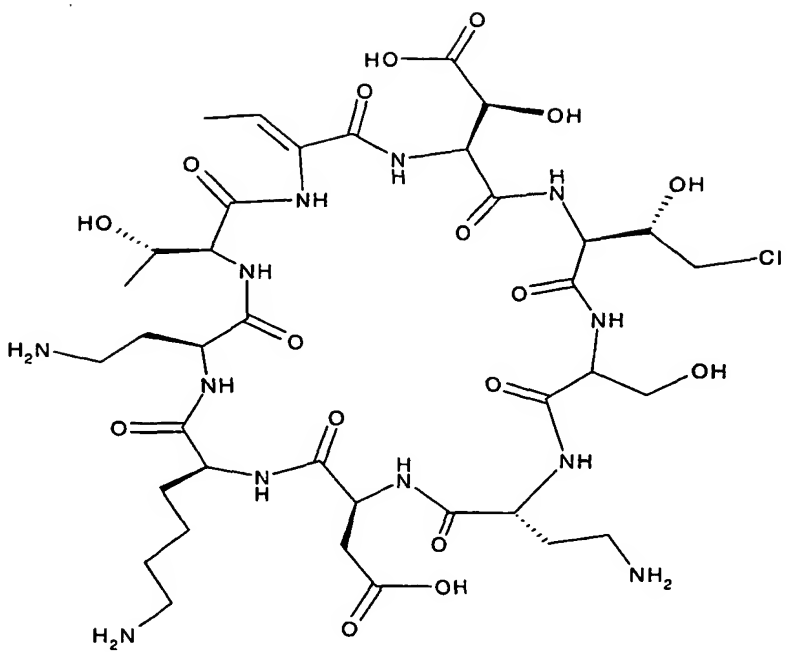
I



II

or a pharmaceutically acceptable salt, hydrate or solvate thereof.

3. The process of Claim 1 wherein said pseudomycin natural product is selected from the group consisting of pseudomycin A, A', B, B', C, and C'.



or a pharmaceutically acceptable salt, hydrate or solvate thereof.

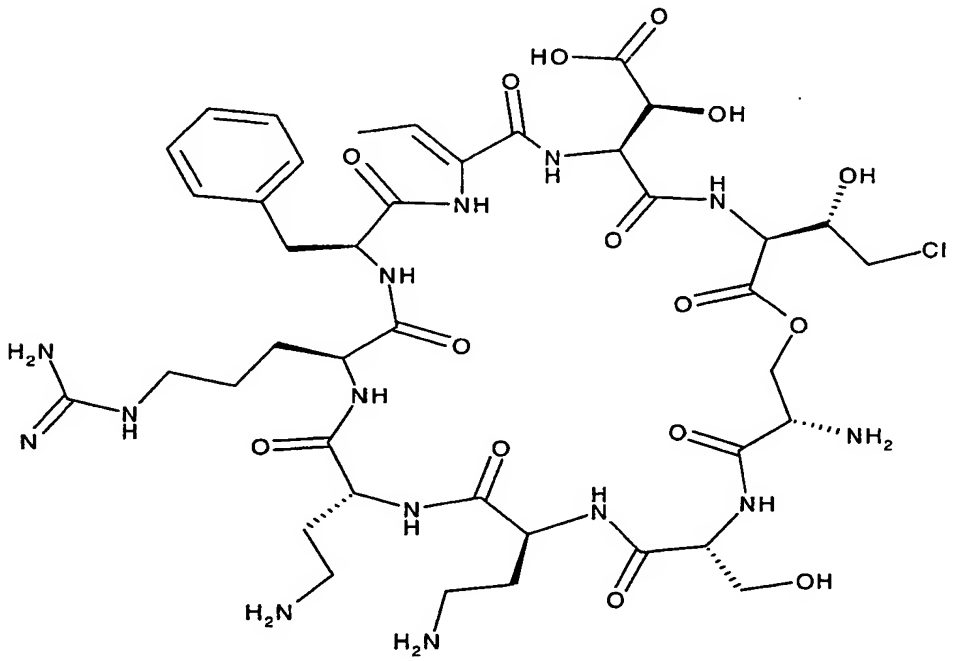
6. A pseudomycin nucleus prepared by reacting a pseudomycin natural product with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase.

7. The pseudomycin nucleus of Claim 6 wherein said pseudomycin natural product is selected from the group consisting of pseudomycin A, A', B, B', C, and C'.

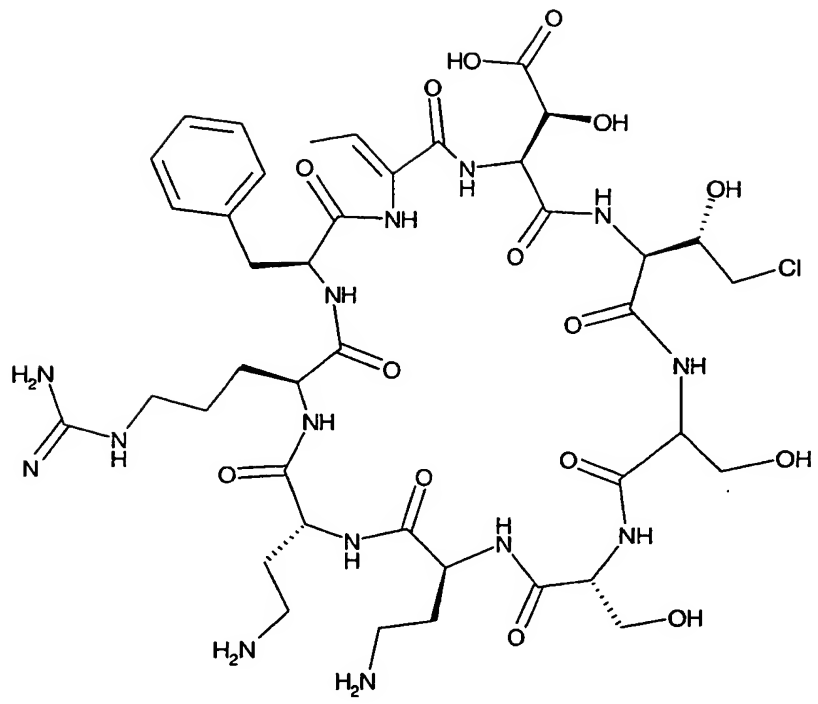
8. A process for deacylating an N-acyl side-chain of a syringomycin natural product comprising the step of

reacting a syringomycin natural product with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce a syringomycin nucleus.

9. The process of Claim 7 wherein said syringomycin nucleus is represented by either structure III or IV



III



IV

or a pharmaceutically acceptable salt, hydrate or solvate thereof.

10. A syringomycin nucleus prepared by reacting a syringomycin natural product with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase.

11. A compound having the following structure

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

COHEN, Cherles E TMT
ELI LILLY AND COMPANY
Lilly Corporate Center
Indianapolis Indiana 46285
ETATS-UNIS D'AMERIQUE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	13.11.2001
Applicant's or agent's file reference X-11650	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/15018	08/06/2000	15/07/1999

Applicant
ELI LILLY AND COMPANY

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits her with the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Articl 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Cardenas, C Tel.+31 70 340-3370

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
X-11650		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/15018	08/06/2000	15/07/1999
International Patent Classification (IPC) or national classification and IPC C07K7/06		
Applicant ELI LILLY AND COMPANY		

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2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

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   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/01/2001	13.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Groenendijk, M Telephone No. +31 70 340 3715

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/15018

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21	as originally filed				
Claims, No.:					
4,5 (part),11 (part), 12	as originally filed				
1-3,5 (part),6-10, 11 (part)	as received on	12/07/2001	with letter of	12/07/2001	

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
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   - ☐ *filed together with the international application in computer readable form.*
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
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4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-12
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-7
	No:	Claims	8-12
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following document/s/:

D1:FEBS Letters,, 355(1994), 96-100
D2:Agric.Biol.Chem., 53(2),1989, 497-504
D3:EP-A-0460882

I.NOVELTY

In view of the available prior art the claims 1-12 are considered to be novel under Art.33(2) PCT.

II.INVENTIVE STEP

1)The closest prior art is considered to be D1, disclosing peptides of the so-called pseudomycin and syringomycin groups and their use as antibiotics.
2)The present application differs from said prior art essentially therein that said compounds are enzymatically deacylated in order to provide the cyclic nucleus which can be used to synthesize sidechain analogs.
3)The problem to be solved may therefore be considered to be the provision of intermediate products of pseudomycin and syringomycin antibiotics as means for preparing sidechain analogs.
4)The solution is a process as defined in claim 1 wherein the sidechain is enzymatically removed by an ECB or polymixin deacylase.
5)It is true that ECB and polymixin deacylase were already known to be able to hydrolyse, in addition to echinocandin and polymixin type of compounds, also related lipopeptides or fatty acid-amino acid adducts (e.g., see D2 and D3).
However the use of said enzymes for the present deacylation has not been indicated or suggested and due to the different structures of the present compounds it could not be expected that the present compounds would actually be properly deacylated, which view is supported by the fact that only selected enzymes appear to be suitable (see description page 9, lines 7-8).
5)Consequently an inventive step can be acknowledged for a process (and the intermediate compounds) as defined in claim 1 as far as it solves the problem posed.

However, as indicated in the previous paragraph, it cannot be predicted whether certain enzymes actually can hydrolyse the present compounds. At present only Pseudomycin A has been demonstrated to be hydrolysed. In order to acknowledge an inventive step to a process for deacylating Syringomycins, which compounds have a different surrounding of the site of hydrolysation, and the obtained intermediate products, that is, the subject-matter of the claims 8-12, additional experimental data should have been filed. In the absence of said data the subject-matter of the claims 8-12 is considered not to solve the problem posed and hence no inventive step can be acknowledged for said claims under Art.33(3) PCT.

Re Item VII

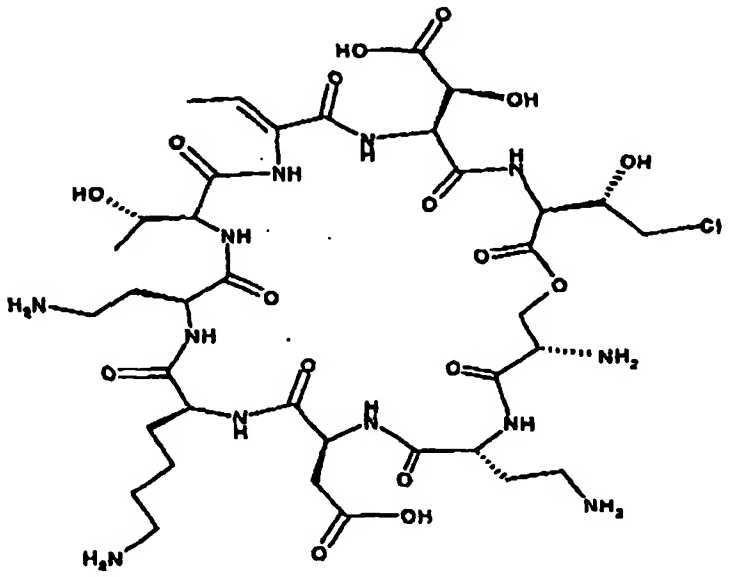
Certain defects in the international application

In several positions in the description the application contains the expression "incorporated by reference". However the application should, regarding the essential features of the invention, be self-contained, that is, capable of being understood without reference to any other document. Consequently said expression should have been deleted from the description and, if necessary, said subject-matter by reference should expressly have been incorporated into the description, subject to the restrictions under the Articles 19(2) and 34(2)(b) PCT (see PCT Guidelines CII, 4.17-4.18).

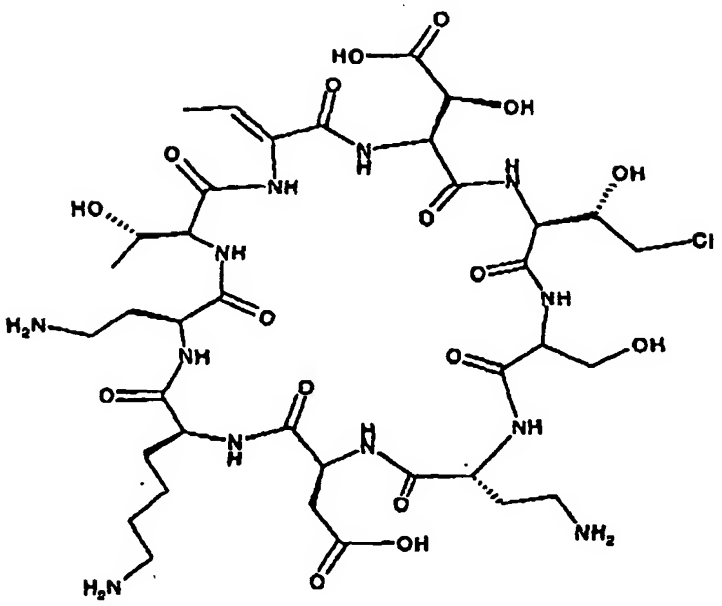
WE CLAIM:

1. A process for deacylating an N-acyl side-chain of a pseudomycin comprising the step of reacting a pseudomycin with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce a pseudomycin nucleus.

2. The process of Claim 1 wherein said pseudomycin nucleus is represented by either structure I or II



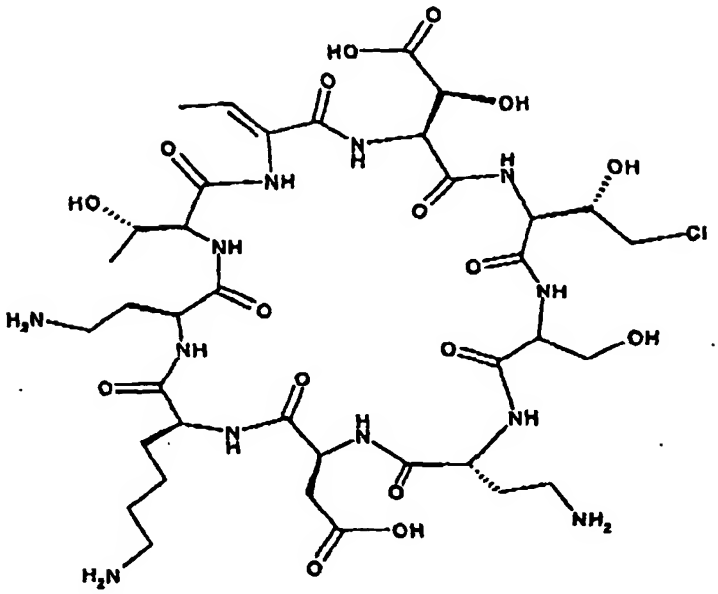
I



II

or a pharmaceutically acceptable salt, hydrate or solvate thereof.

3. The process of Claim 1 wherein said pseudomycin is selected from the group consisting of pseudomycin A, A', B, B', C, and C'.



or a pharmaceutically acceptable salt, hydrate or solvate thereof.

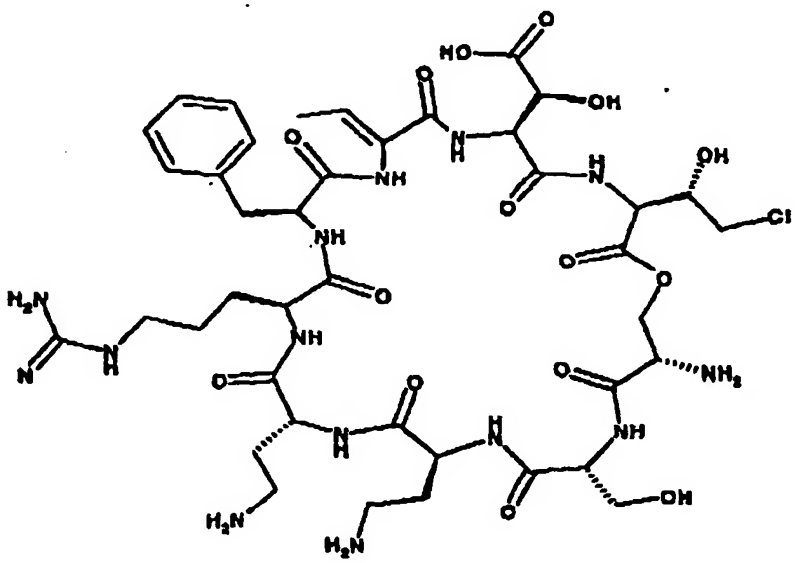
6. A pseudomycin nucleus prepared by reacting a pseudomycin with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase.

7. The pseudomycin nucleus of Claim 6 wherein said pseudomycin is selected from the group consisting of pseudomycin A, A', B, B', C, and C'.

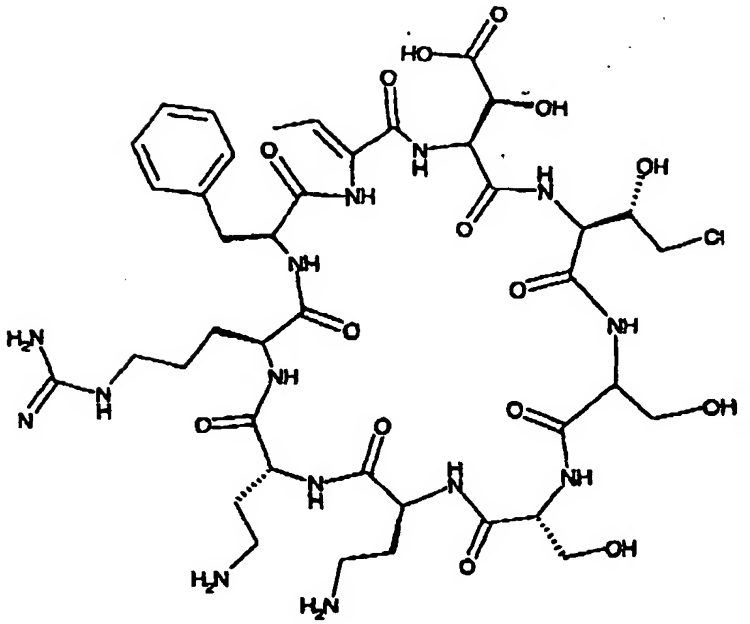
8. A process for deacylating an N-acyl side-chain of a syringomycin comprising the step of

reacting a syringomycin ~~natural product~~ with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce a syringomycin nucleus.

9. The process of Claim 7 wherein said syringomycin nucleus is represented by either structure III or IV



III



IV

or a pharmaceutically acceptable salt, hydrate or solvate thereof.

10. A syringomycin nucleus prepared by reacting a syringomycin with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase.

11. A compound having the following structure

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/US 00/15018

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K7/06 C07K7/64 C12P21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, BIOSIS, MEDLINE, SCISEARCH, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A BALLIO ET AL: "Novel bioactive lipodepsipeptides from Pseudomonas syringae: the pseudomycins" FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 355, no. 1, 21 November 1994 (1994-11-21), pages 96-100, XP002125309 ISSN: 0014-5793 cited in the application the whole document --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2000	26/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. .ational Application No
PCT/US 00/15018

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
A	N FUKUCHI ET AL: "Isolation and structural elucidation of syringostatins, phytotoxins produced by Pseudomonas syringae pv. syringae Lilac isolate" JOURNAL OF THE CHEMICAL SOCIETY, PERKIN TRANSACTIONS 1,GB,CHEMICAL SOCIETY. LETCHWORTH, vol. 7, 1992, pages 875-880, XP002125311 ISSN: 1470-4358 the whole document ---	1-12
A	YASUDA E.A.: "Polymixin acylase: an enzyme causing intramolecular N2-N6 acyl ttransfer in N-monooctanoyl-L-lysine" AGRIC.BIOL.CHEM., vol. 53, no. 12, 1989, pages 3245-3249, XP002149547 cited in the application the whole document ---	1-12
A	KIMURA E.A.: "Polymixin acylase: purification and characterization, with special reference to broad substrate specificity" AGRIC.BIOL.CHEM., vol. 53, no. 2, 1989, pages 497-504, XP002149548 cited in the application the whole document ---	1-12
A	EP 0 460 882 A (LILLY CO ELI) 11 December 1991 (1991-12-11) the whole document -----	1-12

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ..ational Application No
PCT/US 00/15018

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0460882 A	11-12-1991	AT 140728 T	15-08-1996
		CA 2043762 A	08-12-1991
		DE 69121018 D	29-08-1996
		DE 69121018 T	19-12-1996
		DK 460882 T	26-08-1996
		ES 2089133 T	01-10-1996
		GR 3021292 T	31-01-1997
		HU 215232 B	30-11-1998
		IE 75885 B	24-09-1997
		IL 98349 A	26-05-1995
		JP 2994486 B	27-12-1999
		JP 4228072 A	18-08-1992
		KR 169994 B	01-02-1999
		US 5573936 A	12-11-1996

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
X-11650		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/ 15018	08/06/2000	15/07/1999
Applicant		
ELI LILLY AND COMPANY		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

PROCESS FOR DEACYLATION OF LIPODEPSIPEPTIDES

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/15018

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7: C07K7/06 C07K7/64 C12P21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, BIOSIS, MEDLINE, SCISEARCH, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A BALLIO ET AL: "Novel bioactive lipodepsipeptides from Pseudomonas syringae: the pseudomycins" FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 355, no. 1, 21 November 1994 (1994-11-21), pages 96-100, XP002125309 ISSN: 0014-5793 cited in the application the whole document --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2000	26/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Groenendijk, M

3

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	N FUKUCHI ET AL: "Isolation and structural elucidation of syringostatins, phytotoxins produced by Pseudomonas syringae pv. syringae Lilac isolate" JOURNAL OF THE CHEMICAL SOCIETY, PERKIN TRANSACTIONS 1,GB,CHEMICAL SOCIETY. LETCHWORTH, vol. 7, 1992, pages 875-880, XP002125311 ISSN: 1470-4358 the whole document ---	1-12
A	YASUDA E.A.: "Polymixin acylase: an enzyme causing intramolecular N2-N6 acyl ttransfer in N-monooctanoyl-L-lysine" AGRIC.BIOL.CHEM., vol. 53, no. 12, 1989, pages 3245-3249, XP002149547 cited in the application the whole document ---	1-12
A	KIMURA E.A.: "Polymixin acylase: purification and characterization, with special reference to broad substrate specificity" AGRIC.BIOL.CHEM., vol. 53, no. 2, 1989, pages 497-504, XP002149548 cited in the application the whole document ---	1-12
A	EP 0 460 882 A (LILLY CO ELI) 11 December 1991 (1991-12-11) the whole document -----	1-12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna[redacted]al Application No
PC[redacted] 00/15018

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0460882 A	11-12-1991	AT 140728 T	15-08-1996
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		IE 75885 B	24-09-1997
		IL 98349 A	26-05-1995
		JP 2994486 B	27-12-1999
		JP 4228072 A	18-08-1992
		KR 169994 B	01-02-1999
		US 5573936 A	12-11-1996

FEBS Letters 355 (1994) 96–100

FEBS 14792

Novel bioactive lipodepsipeptides from *Pseudomonas syringae*: the pseudomycins

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Abstract The covalent structure and most of the stereochemistry of the pseudomycins, bioactive metabolites of a transposon-generated mutant of a *Pseudomonas syringae* wild-type strain proposed for the biological control of Dutch elm disease, have been determined. While two pseudomycins are identical to the known syringopeptins 25-A and 25-B, pseudomycins A, B, C, C' are new lipodepsinonapeptides. For all of these the peptide moiety corresponds to L-Ser-D-Dab-L-Asp-L-Lys-L-Dab-L-aThr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl) with the terminal carboxyl group closing a macrocycle ring on the OH group of the N-terminal Ser. This is in turn *N*-acylated by 3,4-dihydroxytetradecanoate in pseudomycin A, by 3-hydroxytetradecanoate in pseudomycin B, by 3,4-dihydroxyhexadecanoate in pseudomycin C, and by 3-hydroxyhexadecanoate in pseudomycin C'. Some preliminary data on the biological activity of pseudomycin A are reported.

Key words: Phytotoxin; Lipodepsipeptide; Pseudomycin; Syringomycin; *Pseudomonas syringae*

1. Introduction

Pseudomycins are antifungal metabolites produced in elevated amounts by *Pseudomonas syringae* MSU 16H [1] a transposon-generated mutant of a wild-type strain that has attracted interest for its ability to confer a greater protection than the wild-type strain in elms infected with *Ceratocystis ulmi*, the causal agent of Dutch elm disease [2]. Recently the individual pseudomycins have been isolated and their structures have been partially characterized [1]. The aim of our research on pseudomycins was to complete the study of their structure and to investigate their biological properties. Four of them turned out to be new lipodepsinonapeptides related to syringomycins [3,4], syringotoxin [5,6] and syringostatins [7], a group of antimicrobial and phytotoxic compounds produced by different isolates of *P. syringae* pv. *syringae*. This structural relation prompted a comparison of the biological properties of pseudomycins with those of other lipodepsinonapeptides. The preliminary results (details will be published elsewhere) of some biotests carried out with pseudomycin A, the major pseudomycin, in comparison with syringomycin E [3], a lipodepsinonapeptide extensively investigated for its biological activities [8], are included in this paper.

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Abbreviations: TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment mass spectrometry; TBDMS, *t*-butyldimethylsilyl; Asp(3-OH), 3-hydroxyaspartic acid; Thr(4-Cl), 4-chlorothreonine; Dab, 2,4-diaminobutyric acid; Dhb, dehydro-2-aminobutyric acid; aThr, allothreonine; 2D NMR, two-dimensional nuclear magnetic resonance; NOE, nuclear Overhauser effect; TPPI, time proportional phase increment; FID, free induction decay; NOESY, nuclear Overhauser effect correlated spectroscopy; TOCSY, total correlated spectroscopy.

This paper is dedicated to the memory of our much missed colleague and friend Giampaolo Nitti.

2. Materials and methods

2.1. Microbiological methods

The *P. syringae* MSU 16H strain is an elm tree acclimated transposon (Tn 903) generated mutant of the wild type strain MSU 174. It is equivalent to MSU strain 206 previously described in [2]. It was grown in still culture under conditions reported in [5]. Antifungal activity was assayed with *Rhodotorula pilimanae* [9].

2.2. Preparation of pseudomycins

After 9–10 days growth at 25°C, the bioactive metabolites were extracted and partially purified according to Bidwai et al. [10], and finally fractionated by reverse phase HPLC on an Aquapore RP300 column (4.6 × 250 mm, 7 μm ID, Applied Biosystems) using a Beckman System Gold 126 instrument under conditions described in [11]. Individual peaks were freeze-dried, quantitated by amino acid analysis after HCl hydrolysis, and assayed for activity toward *R. pilimanae*.

2.3. Analytical methods

Amino acids were analyzed as reported in [11], except that an Eppendorf-Biotronik LC 3000 analyzer was used; some analyses were also performed by GC-MS after transformation into TBDMS derivatives [12]. The chirality of amino acid residues was determined by Marfey's method [13]. Peptide sequences were obtained by automated Edman degradation using an Applied Biosystems model 476A sequencer. Samples were spotted on Problott membrane (Applied Biosystems) and sequenced with a Blott Cartridge (Applied Biosystems).

FAB-MS spectra were recorded on a VG ZAB 2SE instrument equipped with a cesium gun operating at 25 kV, 2 μA. Samples dissolved in 5% acetic acid were directly loaded onto the probe tip coated with glycerol/thioglycerol 1:1 (v/v).

NMR spectra were run at 27°C on a Bruker AMX600 instrument operating at 600.13 MHz. Samples (1 mg) were dissolved in 0.8 ml of either D_2O, or H_2O/D_2O 90:10, at pH 4.8. 2D NMR experiments were performed in the phase-sensitive mode with TPPI phase cycle [14] typically using 2K of memory for 512 increments. The number of scans were optimized in order to obtain a satisfactory signal-to-noise ratio. Total Correlation experiments (TOCSY) were performed using the MLEV-17 spinlock composite pulse sequence [15,16] with a typical mixing time ranging from 80 to 120 ms (relayed) in order to observe either direct or remote connectivities. NOE dipolar correlated 2D spectra were obtained using the NOESY pulse sequence [17]. The mixing

time for the magnetization exchange ranged from 60 to 220 ms. Data were processed on a microVax II graphics workstation by the 'TRITON' 2D NMR software of R. Boelsen and G. Vuister, kindly provided by Prof. R. Kaptein of Utrecht University. FIDs were weighted by a sinebell apodization function shifted typically $\pi/3$ in both dimensions. In all homonuclear 2D experiments, a matrix 1,024 × 1,024 in the phase-sensitive mode was thus obtained with a digital resolution of ≈3 Hz/point. A baseline correction was carried out in both dimensions using a polynomial fit. ^{13}C-^{1}H heteronuclear correlations were obtained in the reverse-detection mode on the AMX600 Bruker instrument (1K × 512 w).

2.4. Chemical methods

The lactone ring hydrolysis was obtained by a 3 h incubation at 37°C with 50 mM ammonium bicarbonate.

2.5. Enzymatic hydrolyses

Lipodepsipeptides (250 μg) dissolved in 0.1 M ammonium bicarbonate (150 μl) pH 8.0, were incubated at 37°C for 5 h with TPCK-trypsin (Worthington Biochemicals Co.) using an enzyme/substrate ratio of 1:30. After lyophilization the hydrolysis products were fractionated by HPLC as described under 2.2. Elution was performed with a solvent gradient obtained by mixing 0.2% TFA in water with 70% acetonitrile containing 0.2% TFA, and with a flow rate of 0.8 ml/min. The main peaks were freeze-dried and the samples were analyzed by FAB-MS and Edman degradation.

2.6. Biotests

Tobacco leaf assay was performed as in [18]; the other assays were carried out as in [19].

3. Results and discussion

Reverse phase HPLC of a *P. syringae* MSU 16H extract partially purified according to Bidwai et al. [10] produced an elution pattern of the same type observed with syringomycin- and syringotoxin-producing strains (Fig. 1) [11]. Several peaks appeared in the region where the lipodepsinonapeptides are eluted, followed by two more hydrophobic peaks emerging from the column at higher acetonitrile-isopropanol concentration. FAB-MS and amino acid analyses (see below) of the substances isolated from the six more relevant peaks indicated that four corresponded to the previously described [1] pseudomycin A, B, C and D, another (C′) could be a further pseudomycin, and the sixth (D′) presumably corresponded to the minor component previously found as a contaminant of purified D and labelled D′ [1]. The amino acid composition (see below) clearly indicated that the substances isolated from peaks A, B, C and C′ were different from known *P. syringae* metabolites, while those of compounds in peaks D and D′ compared well with those of the two syringopeptins 25-A and 25-B [11]. The identity of the two compounds from peaks D and D′ with the two syringopeptins was proved by the same MH^+ values, absorbance at 280 nm, HPLC elution times, and detailed ^{1}H-NMR data (not reported); the name pseudomycin should thus be abolished for the two more hydrophobic metabolites.

The complete structure of compounds contained in peaks A, B, C and C′ was elucidated by the use of 2D NMR, FAB-MS and chemical and enzymatic degradations carried out on microquantities. Pseudomycin A, a relatively abundant component, was at first investigated. Amino acid determination, both by conventional ion-exchange chromatography and by GC-MS after derivatization with *N*-methyl-*N*-TBDMS-trifluoroacetamide [12], showed the presence of one mol each of Ser, aThr, Asp, Asp(3-OH), Thr(4-Cl), Lys, and two mol of Dab. The methods commonly used for 2D NMR studies reached the same conclusion and furthermore identified a Z-Dhb residue and the 3,4-dihydroxytetradecanoyl moiety; the chemical shifts and assignements of ^{1}H and ^{13}C-NMR spectra are reported in Table 1. All amino acid residues, with the exception of one Dab, had the L configuration.

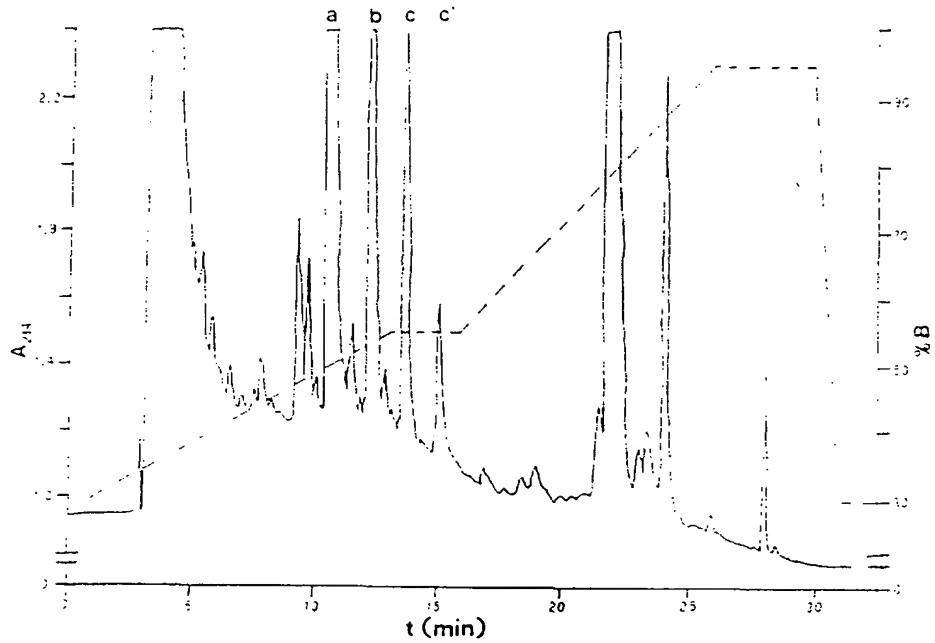

Fig. 1. Reverse-phase HPLC of the metabolites from *Pseudomonas syringae* strain MSU 16H. The letters A, B, C and C′ indicate the pseudomycins. The two main hydrophobic peaks are SP_{25}-A and SP_{25}-B.

The possibility that pseudomycin A is a new lipodepsinonapeptide got support from the occurrence in the molecule of nine amino acid residues and a long chain hydroxyacyl, from the difference of 18 mass units between the calculated sum of the residues and the molecular weight found by FAB-MS (MH^+ 1,223–1,225; the doublet indicates the presence of one chlorine atom), from the observed addition of one mol of water (MH^+ 1,241–1,243) by treatment with ammonium bicarbonate (followed by substitution of chlorine with OH: MH^+ 1,223 singlet), and from the absence of a free N-terminus. FAB-MS of a sample treated with ammonium bicarbonate produced a fragmentation pattern in agreement with the following partial

Table 1
^1H and ^{13}C chemical shifts and relative assignments for pseudomycin A.

δ ppm ^1H	Assignment	Residue	δ ppm ^{13}C observed	δ ppm ^{13}C literature
2.58	2 CH	Fatty acid	38.5	43.9 (20)
2.53	2′ CH		38.5	43.9 (20)
3.95	3 CH		72.2	69.7 (20)
3.63	4 CH		74.6	69.7 (20)
1.63	5′ CH		32.5	37.8 (20)
1.57	5 CH		32.5	37.8 (20)
1.46	6 CH		25.5	26.2 (20)
1.43	6′ CH		25.5	26.2 (20)
1.35	7–11 CH		29.3	29.9 (20)
1.35	12 CH		32.2	32.4 (20)
1.33	13 CH		22.7	14.3 (20)
0.9	14 CH		14.3	14.3 (20)
8.90	NH	Ser	/	/
4.76	α CH	Ser	58.1	56.9 (21)
4.39	β CH	Ser	61.8	60.8 (21)
8.83	NH	Dab 1	/	/
4.32	α CH	Dab 1	52.3	52.7 (20)
2.25	β CH	Dab 1	28.2	29.2 (20)
3.22	γ CH	Dab 1	37.5	37.9 (20)
3.15	γ′ CH	Dab 1	37.5	37.9 (20)
8.46	NH	Asp	/	/
4.22	α CH	Asp	55.3	52.7 (21)
2.87	β′ CH	Asp	37.0	37.1 (21)
2.73	β CH	Asp	37.0	37.1 (21)
8.36	NH	Lys	/	/
4.15	α CH	Lys	55.4	54.8 (21)
2.02	β CH	Lys	28.2	30.2 (21)
1.36	γ CH	Lys	27.0	21.9 (21)
1.24	γ′ CH	Lys	27.0	21.9 (21)
1.73	δ CH	Lys	23.0	26.7 (21)
2.99	ε CH	Lys	39.8	39.5 (21)
9.19	NH	Dab 2	/	/
4.59	α CH	Dab 2	51.7	52.7 (20)
2.33	β CH	Dab 2	29.8	29.2 (20)
2.18	β′ CH	Dab 2	29.8	29.2 (20)
3.15	γ CH	Dab 2	37.2	37.9 (20)
8.48	NH	Thr	/	/
4.20	α CH	Thr	61.7	61.0 (21)
4.10	β CH	Thr	66.8	66.6 (21)
1.35	γ CH	Thr	20.3	20.0 (21)
9.65	NH	Dhb	/	/
6.87	β CH	Dhb	129	134 (20)
1.77	γ CH	Dhb	13.6	13.3 (20)
7.85	NH	OHAsp	/	/
5.02	α CH	OHAsp	57.5	57.0 (20)
4.83	β CH	OHAsp	73.5	72.3 (20)
8.72	NH	ClThr	/	/
5.14	α CH	ClThr	55.3	55.8 (20)
4.53	β CH	ClThr	72.1	72.3 (20)
3.62	γ′ CH	ClThr	45.2	45.8 (20)
3.55	γ CH	ClThr	45.2	45.8 (20)

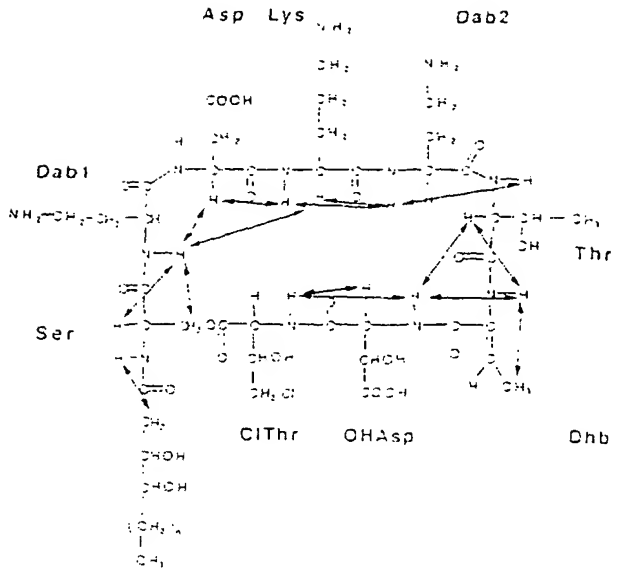

Fig. 2. Chemical structure of Pseudomycins A ($n = 9$) and C ($n = 11$). Only the short range NOE contacts have been reported.

sequence: Asp-Lys-Dab-aThr-Dhb-Asp(3-OH)-Thr(4-OH), where Thr(4-OH) arises from Thr(4-Cl) at basic pH. The occurrence of an L-lysine residue prompted us to cleave pseudomycin A by trypsin. After incubation with the enzyme, the solution was fractionated by reverse phase HPLC. The fragment present in the main hydrophilic peak corresponded to the C-terminal part of the molecule, as ascertained by automated Edman degradation which yielded a Dab residue on the first cycle and a Thr residue on the second; the sequence determination was stopped in correspondence of the dehydro-amino acid [20]. Treatment of the same fragment by a modified Marfey's procedure [13] allowed us to assign the L-configuration to the Dab residue adjacent to Lys and consequently the D-configuration to the other Dab residue. The FAB-MS spectrum of a prominent more hydrophobic peak showed the pseudomolecular ion (MH^+ 691) expected for the rest of the molecule, namely for 3,4-dihydroxytetradecanoyl-(Dab,Ser)-Asp-Lys-OH. The complete sequence of the tetrapeptide moiety and the site of acylation emerged from NMR spectra of pseudomycin A (see Table 1). The chirality of carbons 3 and 4 of the fatty acid moiety has not yet been determined. The otherwise complete structure of pseudomycin A is reported in Fig. 2, where the arrows indicate the short range strong NOE contacts which have allowed us to elucidate, independently from the chemical approach, the amino acid sequence, as well as the position and the type of closure of the macro ring. A number of long range NOE contacts have also been identified; these proximities, together with available information about the chirality of the amino acid residues, are prerequisites for the determination of the solution structure of this molecule.

Pseudomycins B (MH^+ 1,207–1,209), C (MH^+ 1,251–1,253) and C′ (MH^+ 1,235–1,237) are closely related to pseudomycin A. In fact, amino acid composition, and fragmentation observed in the FAB-MS spectra after lactone opening with ammonium bicarbonate gave identical results for all four pseu-

Table 2
Structure of pseudomycins (PSs), syringostatins (SSs), syringotoxin (ST) and syringomycins (SRs).

Me(CH$_2$)$_n$-CH(X)-CH(OH)-CH$_2$-CO-L-Ser-aa$_1$-aa$_2$-aa$_3$-aa$_4$-aa$_5$-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)

	n	X	aa$_1$	aa$_2$	aa$_3$	aa$_4$	aa$_5$
PSs A, C	9,11	OH	D-Dab	L-Asp	L-Lys	L-Dab	L-aThr
PSs B, C	9,11	H					
SSs	7,9	H, OH	D-Dab	L-Dab	D-Hse	L-Orn	L-aThr
ST	9	H	D-Dab	Gly	D-Hse	L-Orn	L-aThr
SRs	5,7,9	H	D-Ser	D-Dab	L-Dab	L-Arg	L-Phe

domycins. Thus, very likely they differ only for the long chain acyl group: their MH$^+$ values suggest that the nonapeptide moiety is acylated in pseudomycin B by 3-monohydroxytetradecanoate, in pseudomycin C by 3,4-dihydroxyhexadecanoate, and in pseudomycin C′ by 3-monohydroxyhexadecanoate. The position of the four pseudomycins in the reverse phase HPLC elution pattern is consistent with an increased hydrophobicity on passing from A to C′.

As compared to the known lipodepsinonapeptides from *P. syringae* pv. *syringae* (Table 2), the pseudomycins have: (i) the same variety of fatty acids (3-hydroxy and 3,4-dihydroxy) previously found in syringostatins, except that some have a longer aliphatic chain (C_{13}); (ii) an identical N-terminal residue (L-Ser), *N*-acylated by the fatty acid and *O*-acylated by the terminal carboxyl group; (iii) the same C-terminal tripeptide [Dhb-L-Asp(3-OH)-L-Thr(4-Cl)] with the carboxy group closing the lactone ring; (iv) a D-amino acid residue in the second position, similarly to all so far described congeners obtained for isolates of *P. syringae* pv. *syringae* [21]; (v) the third and the fourth residues with the L-configuration, while in their congeners either one or the other has the D-configuration [21]; (vi) the fifth residue correspondent to a basic L-amino acid residue (Dab), as found in syringomycins (Arg) [3,4], syringotoxin (Orn) [5,6], syringostatins (Orn) [7]. The occurrence of L-Asp in the peptide moiety of the pseudomycins is a novel feature of the lipodepsinonapeptides which might affect their conformation and biological properties.

Table 3
A comparison between some biological activities of 20 μM syringomycin-E (SR) and 20 μM pseudomycin A (PS).

	SR	PS
Induction of necrosis in tobacco leaves	+ [18]	+
Inhibition of proton extrusion promoted by fusicoccin in maize roots	++ [19]	+
Stimulation of ATP hydrolysis in right-side-out plasma membrane vesicles from maize roots	+ [19,24]	++
Inhibition of ATP hydrolysis in inside-out plasma membrane vesicles from maize roots	++ [19,24]	±
Inhibition of proton translocation in inside-out plasma membrane vesicles from maize roots	+ [19]	±
Dissipation of the pH gradient in inside-out plasma membrane vesicles from maize roots	++ [19]	±

Preliminary data (Table 3) on the phytotoxicity of pseudomycin A, and on its activity in vitro and in vivo towards some fundamental processes operative at the level of the plant plasma membrane, indicate that the new lipodepsipeptide has a behaviour very similar to that of syringomycin E. At the same molar concentration the pseudomycin is less active than the syringomycin, with the exception of a higher efficiency in stimulating ATP hydrolysis in right-side-out plasma membrane vesicles, this result is compatible with the weaker inhibition of ATP-ase activity.

Acknowledgements: This work has been supported in part by grants of the Italian National Research Council (CNR), special ad hoc programme 'Chimica fine II', subproject 3, and of the Italian Ministry for University and Scientific and Technological Research (MURST); and by NATO Grant 921129 to A.B. Mass spectral data were obtained at Servizio di Spettrometria di Massa del CNR, Università di Napoli 'Federico II'. We thank Dr. Lorenzo Camoni for skilful technical assistance.

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Isolation and Structural Elucidation of Syringostatins, Phytotoxins Produced by *Pseudomonas syringae pv. syringae* Lilac Isolate

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A bacterial strain of *Pseudomonas syringae pv. syringae* isolated from lilac was found to produce a homologous mixture of phytotoxins different from syringomycin and syringotoxin. The toxins were termed syringostatins and the structures of the main components, syringostatins A and B, were determined by 2D-NMR spectroscopy and mass spectrometry. Minor component structures were elucidated from mass/mass spectra.

Pseudomonas syringae pv. syringae is a phytopathogenic bacterium which damages crops, fruit trees and various plants world wide. This bacterium is known to produce at least two different phytotoxins as virulence determinants.[1,2] Syringomycin is produced by the strains from various diseased plants, and syringotoxin is produced by strains from fruit trees. Although long recognized as peptides[3] and known to be toxic to plant cells,[4] little structural information was known until Segre *et al.*[5] reported the partial structure of syringomycin E.

In the 1980s, lilac blight spread through the central parts of Japan, and *Pseudomonas syringae pv. syringae* was isolated as the causative bacterium of the disease.[6] We made initial attempts to isolate phytotoxins produced by the bacterium (designated strain SY12) by monitoring the inhibitory activity to lettuce seedlings. These efforts yielded indole-3-acetic acid as the active substance. Then, we screened further for toxic metabolites in the cultured medium of the bacterium by detecting antimicrobial activity to *Penicillium chrysogenum*.

As a result, we succeeded in isolating peptidal toxins which differed from the known two toxins, syringomycin and syringotoxin. The toxin preparations were composed of several components and termed syringostatins.[7] The structures of the main components have been reported in preliminary form.[8] In this paper, we describe the production, isolation and structural elucidation of the syringostatins in detail.

The production level of syringostatins by strain SY12 was sensitive to culture conditions. We established the optimal conditions to be as follows: A nutrient broth culture (2 cm^3) was seeded into modified potato–dextrose–broth (200 cm^3) in 500 cm^3 capacity Erlenmeyer flasks. The flasks were incubated without shaking at 26.5 °C for 7 days. The quantity of the toxin produced was estimated by antifungal activity to *Penicillium chrysogenum*.

The toxins were labile under basic condition and lost antifungal activity completely by alkaline treatment. Therefore, the entire isolation procedure was performed under neutral or acidic conditions. The toxins were isolated by successive XAD-7, Sephadex G-25 and Sephadex LH-20 column chromatography. The final purification was performed by HPLC using a reverse-phase ODS column. The major components, syringostatin A **1** and a mixture of syringostatins B **2** and C **3**, and minor components syringostatins D **4**, E **5**, F **6**, G **7** and H **8** were isolated as shown in Fig. 1. Compound **1** and **2** were further separated by HPLC with another reverse-phase column. The yields were 7.4, 5.5 and 4.5 mg from 9 dm^3 of the broth culture for **1**, **2** and **3**, respectively, and less than 1 mg for the other minor components.

The FAB-MS spectra of all the syringostatins revealed $(M + H)^+$ ions around *m/z* 1200. Their isotopic patterns could be divided into two types; one suggested the presence of a chlorine atom and the other was normal. The mass spectral features are summarized in Table 1 and Fig. 2. The high resolution FAB-MS spectra of **1**, **2** and **4** confirmed the above estimation and suggested their molecular formulae as $C_{50}H_{87}ClN_{12}O_{18}$, $C_{50}H_{87}ClN_{12}O_{19}$ and $C_{50}H_{88}N_{12}O_{20}$, respectively.

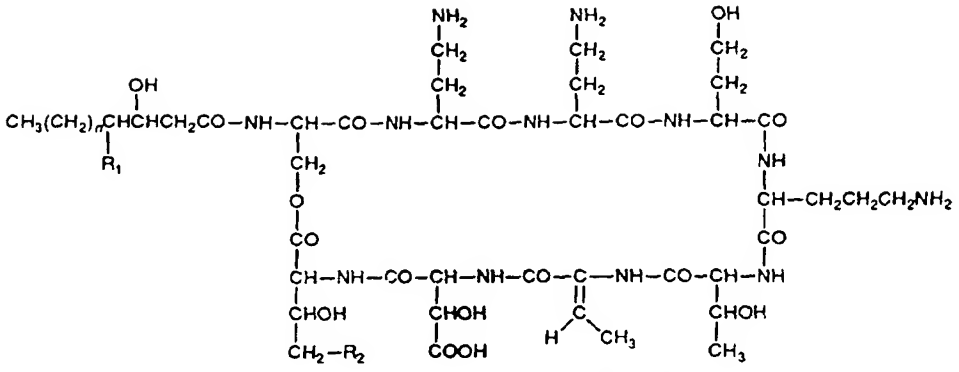
Amino acid analyses of **1** and **2** showed that each had one mole of serine (Ser),* allothreonine (alloThr), ornithine (Orn), two moles of 2,4-diaminobutanoic acid (Dab) and some unknown amino acids.

The ^1H NMR spectra of **1** and **2** showed only broad signals in D_2O and in [$^2H_6$]-DMSO. The proton signals containing amide NH signals resonated sharply in a mixture of CD_3CN and H_2O (7:2 v/v). The COSY spectra and HOHAHA spectra indicated the presence of homoserine (homoSer), β-hydroxyaspartic acid (HyAsp) and 2-aminobut-2-enoic acid (dehydrothreonine; DhThr) residues and an NH-CHOH-CH_2X unit besides the above amino acid residues determined by the amino acid analyses. Further analyses of COSY spectra established the presence of long chain aliphatic residues in **1** and **2**, and showed that they were 3-hydroxyfatty acid and 3,4-dihydroxyfatty acid residues, respectively. Except for the signals due to the fatty acid residues, the ^1H NMR spectra of **1** and **2** were identical.

Fatty acid analyses of the acid hydrolysates of **1** and **2** by GC-MS indicated the presence of 3-hydroxytetradecanoic acid and 3,4-dihydroxytetradecanoic acid residues in **1** and **2**, respectively.

^{13}C NMR and HMQC spectra of **2** confirmed all of the non-carbonyl carbon assignments, and further indicated that the remaining unit was 2-amino-4-chloro-3-hydroxybutanoic acid (4-chlorothreonine; ClThr).[9] Because of the higher chemical shift (δ_C 46.5) for the C-4 carbon (for which the protons resonated at δ_H 3.51 and 3.56) the chlorine atom should be attached to the C-4 carbon. Similarly the presence of this unique amino acid in **1** was established. The above units could explain all of the signals in ^1H and ^{13}C NMR spectra of **1** and **2**, and satisfied the molecular formula suggested by the high resolution FAB-MS spectra when considering one dehydration in the molecule. Thus, the molecular formulae of **1** and **2** indicated cyclic peptides composed of one hydroxyfatty acid and nine amino acid residues.

* *Abbreviations:* serine (Ser), allothreonine (alloThr), ornithine (Orn), 2,4-diaminobutanoic acid (Dab), homoserine (homoSer), β-hydroxyaspartic acid (HyAsp), dehydrothreonine (DhThr) and 4-chlorothreonine (ClThr).



		n	R_1	R_2
Syringostatin	A	9	H	Cl
	B	9	OH	Cl
	D	9	OH	OH
	E	7	H	Cl
	F	9	H	OH
	H	11	OH	Cl

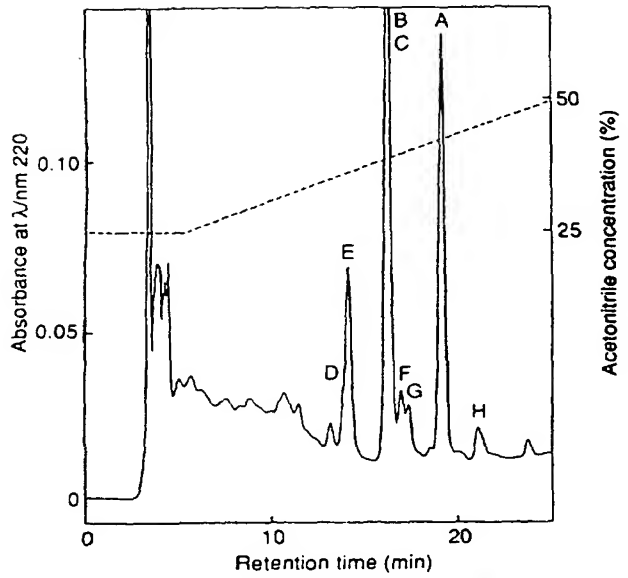


Fig. 1 Chromatogram of syringostatins from reversed phase HPLC. Column: SSC-ODS-H-3201 (250 × 8 mm); eluting solvent: 25–50% acetonitrile in 0.1% TFA (linear gradient)

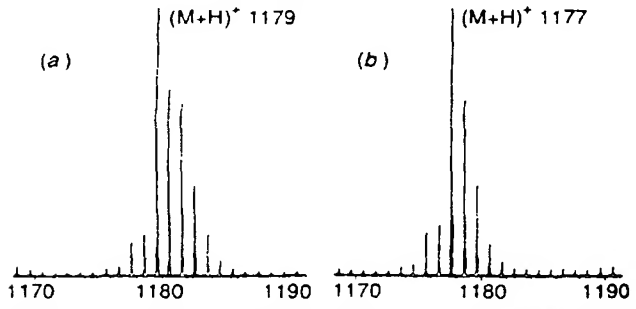


Fig. 2 Isotopic ion patterns of $(M + H)^+$ ions in the FAB mass spectra of syringostatins A **1** (*a*) and D **4** (*b*)

The sequence of these units in **2** was determined by NOESY and HMBC spectra. NOE correlations between α-protons and amide NH protons beyond carbonyl carbons, and two amide NH protons in the neighbouring two residues suggested the sequence from the 3-hydroxy acid residue to ClThr residue as shown in Fig. 3. NOE correlation between the methyl proton and the β-proton in DhThr residue suggested that this residue was (*Z*)-form. The carbon–proton long-range couplings observed in the HMBC spectrum enabled the assignment of all the carbonyl carbons as shown in Table 2 and confirmed the above peptide sequence. Furthermore, the long-range couplings between the β-protons of Ser and carbonyl carbon of ClThr in the HMBC spectrum indicated that the hydroxy group of Ser and the carboxy group of ClThr should form an ester bond. The involvement of the hydroxymethyl group of Ser was confirmed by the lower field shift of the corresponding protons (δ_H: 4.41, 4.52). Similar data were obtained in the spectra of **1**.

The remaining problem in establishing the covalent structures of **1** and **2** was whether the α- or β-carbonyl in the HyAsp residue should be involved in the amide bond with the ClThr residue. This was solved by measuring the chemical shift change of α- and β-protons of HyAsp residue at different pHs. The ^1H NMR spectrum of **2** was measured first in D_2O (pH *ca.* 6.0), then the spectrum was run again after addition of DCl (0.025 mol dm^{-3} DCl), which changed the pH of the solution to *ca.* 3.0. Both the α- and β-protons had similar sharp doublets and similar chemical shifts in this solvent. In 90% H_2O–D_2O (pH *ca.* 6.0), the α-proton gave a double-doublet signal due to the coupling with the amide NH proton. The downfield shift of the β-proton (0.28 ppm) was more sensitive to the pH shift than that of the α-proton (0.14 ppm), showing that the β-carbonyl was the free carboxylic acid and the α-carbonyl was involved in the amide bond. The same result was obtained for **1**. From the above data, the covalent structures of **1** and **2** were determined.

The syringostatins were labile under alkaline condition. Incubation of **1** in 0.1 mol dm^{-3} Tris-HCl buffer (pH 8.5) at 37 °C for half an hour yielded a derivative named SYL-A **9**; further treatment for several hours yielded nearly quantitatively another derivative named SYH-A **10**. In the same way, **2** gave derivatives SYL-B **11** and SYH-B **12**. From the FAB mass spectra, **9** and **11** gave $(M + H)^+$ ions at 1197 and 1213 with ion patterns showing the preservation of the chlorine atom. ^1H NMR spectra of **9** showed hydroxymethyl protons of Ser residue shifted upfield (δ_H 3.78, 3.83). These data indicated that **9** and **11** were linear derivatives formed by hydrolysis of the ester bonds. On the other hand, the derivatives **10** and **12** gave $(M + H)^+$ ions at 1179 and 1195 in FAB mass spectra, respectively, and these ions lost the typical isotopic ion patterns due to a chlorine atom. Therefore, these compounds were generated by replacement of chlorine atoms by hydroxy groups probably through epoxide formation. Fragment ions commonly observed in FAB mass spectra of both **9** and **10** showed many *N*-terminal ions as shown in Fig. 4 and those ions confirmed the proposed

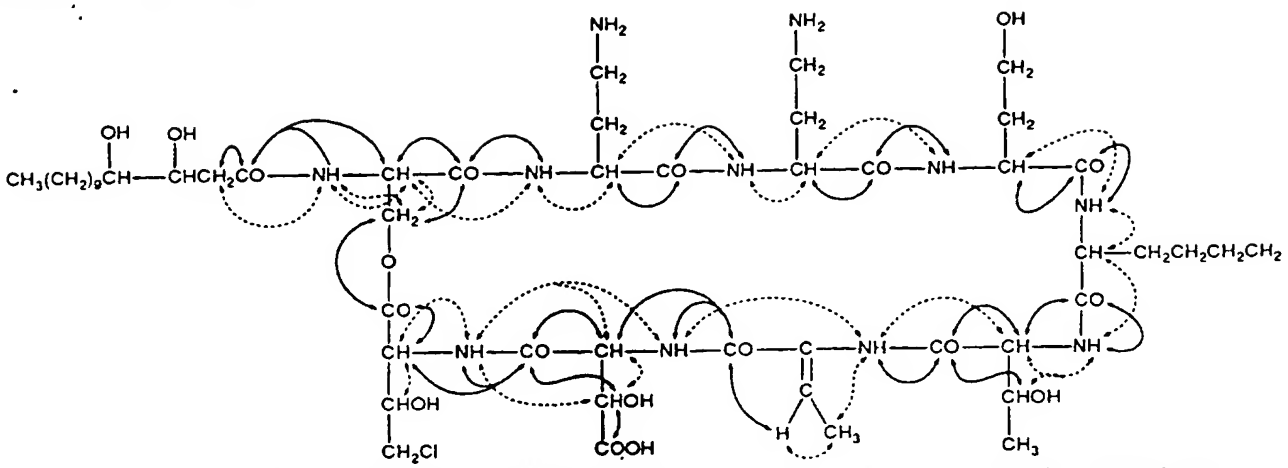


Fig. 3 Structure of syringostatin B 2. The heavy arrows represent the carbon–proton long-range couplings observed in the HMBC spectrum, and the broken arrows represent the NOE correlations observed in the NOESY spectrum.

Table 1 FAB mass spectra of syringostatins

Component names	$(M + H)^+$	Ion patterns	Proposed formula	Mass error (mmu)
Syringostatin A	1179, 5970	chlorine	$C_{50}H_{88}ClN_{12}O_{18}$	−5.7
Syringostatin B	1195, 5890	chlorine	$C_{50}H_{88}ClN_{12}O_{19}$	−8.7
Syringostatin C	1211	chlorine		
Syringostatin D	1177, 6230	normal	$C_{50}H_{89}N_{12}O_{20}$	−8.5
Syringostatin E	1151	chlorine		
Syringostatin F	1161	normal		
Syringostatin G	1211	chlorine		
Syringostatin H	1223	chlorine		

Table 2 ^{13}C and ^1H NMR spectroscopic data for syringostatin B 2[a]

Unit	Carbon	δ_C	δ_H	Unit	Carbon	δ_C	δ_H
A	1	175.1		E	1	174.4	
	2	39.2	2.36 (dd, *J* 10, 15)		2	53.1	4.35 (m)
			2.53 (dd, *J* 3, 15)		3	34.1	2.01 (m)
	3	72.7	3.85 (m)		4	58.8	3.51 m)
	4	75.1	3.48 (m)				3.58 (m)
	5	33.3	1.30 (m)		NH		7.99 (d, *J* 7)
			1.52 (m)	F	1	174.2	
	6	26.4	1.30 (m)		2	53.2	4.47 (ddd, *J* 5, 9, 11)
			1.48 (m)		3	28.9	1.76 (m)
	7–10	30.2					1.99 (m)
	11	29.9	1.30 (m)		4	24.2	1.66 (m)
	12	32.4			5	39.9	2.97 (m)
	13	23.2			NH_2		7.49 (br)[b]
	14	14.3	0.90 (t, *J* 6)		NH		8.25 (d, *J* 9)
B	1	171.8		G	1	172.5	
	2	53.6	4.67 (ddd, *J* 6, 6, 6)		2	61.5	4.18 (dd, *J* 6, 7)
	3	65.4	4.41 (dd, *J* 6, 11)		3	67.6	4.09 (dq, *J* 7, 6)
			4.52 (dd, *J* 6, 11)		4	20.7	1.31 (d, *J* 6)
	NH		8.31 (d, *J* 6)		NH		8.07 (d, *J* 6)
C	1	173.4		H	1	166.2	
	2	52.9	4.29 (ddd, *J* 7, 7, 9)		2	128.8	
	3	29.2	2.08 (m)		3	136.5	6.81 (q, *J* 7)
			2.17 (m)		4	13.8	1.75 (d, *J* 7)
	4	37.6	3.03 (m)		NH		9.31 (s)
	NH_2		7.58 (br)[b]	I	1	171.8	
	NH		8.46 (d, *J* 7)			56.9	5.06 (dd, *J* 2, 9)
D	1	172.9			3	72.4	4.76 (d, *J* 2)
	2	52.2	4.37 (m)		4	175.3	
	3	29.5	2.10 (m)		NH		7.75 (d, *J* 9)
			2.24 (m)	J	1	170.8	
	4	37.6	3.01 (m)		2	55.8	4.87 (dd, *J* 3, 9)
	NH_2		—		3	72.1	4.36 (m)
	NH		8.17 (d, *J* 7)		4	45.8	3.54 (dd, *J* 7, 11)
							3.59 (dd, *J* 6, 11)
					NH		8.12 (d, *J* 9)

[a] Solvent: CD_3CN–H_2O (7:2 v/v). *J* values are given in Hz. [b] Broadened signal.

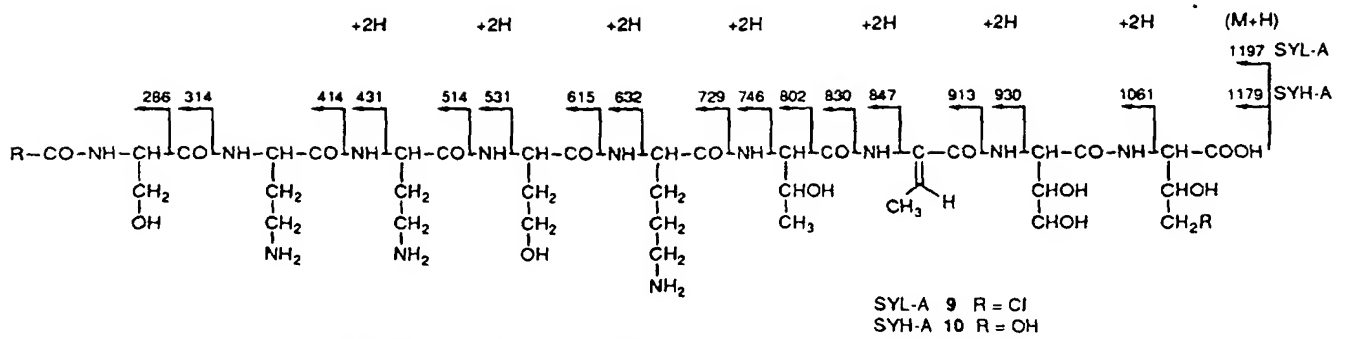


Fig. 4 Fragment ions observed in the FAB mass spectra of SYL-A **9** and SYH-A **10**

sequences of syringostatin A **1**. Similar *N*-terminal fragment ions in the FAB mass spectra of **11** and **12** (all 16 mass units less than those of **9** and **10** respectively) were also consistent with the proposed structure for **2**.

In the FAB mass spectra of **1** and **2**, several fragment ions were observed, but they were not so useful for sequence analyses of these cyclic depsipeptides. Also in the mass/mass spectra, the sequences of **1** and **2** could not be deduced, although more fragment ions were detected than in the FAB mass spectra. On the other hand, the linear derivative such as **9**, **10**, **11** and **12** gave many fragments in the FAB mass spectra, useful for sequence analysis as shown above. But, these were restricted to *N*-terminal fragments, and *C*-terminal fragments were not observed. For **9** and **10**, fragments not observed in the FAB mass spectra were observed in the mass/mass spectra and these could be assigned to *C*-terminal fragments; the data of **10** is shown in Fig. 5 as an example. The *N*- and *C*-terminal fragments were identified according to fragmentation patterns known for linear peptides.[10] The structures of the minor components of the syringostatins were then deduced by FAB mass and mass/mass spectra of the linear derivatives of the minor syringostatins. Fig. 6 shows the fragment ions and the fragmentation patterns of these linear derivatives. The FAB mass and mass/mass spectra of the linear peptides **13** and **14** obtained from **4** and **6**, respectively, were the same as those of **12** and **10**. Therefore, **4** and **6** should have HyThr instead of ClThr in **2** and **1**. The retention times of **13** and **14** in the HPLC were also the same as those of **12** and **10**, respectively. The linear derivative **15** obtained from **5** gave the *N*-terminal fragments which were 28 mass units less than those of **9**, indicating that **5** bears a 3-hydroxydodecanoyl residue instead of 3-hydroxytetradecanoyl in **1**. The derivative **16** obtained by hydrolysis of **8** showed $(M + H)^+$ ion at *m/z* 1241, which was 28 mass units more than that of **11**, and the C-terminal fragments of **16** were all identical to those of **11**. Therefore the difference between **8** and **2** was in the acid residue, with **8** having the 3,4-dihydroxyhexadecanoyl residue. Thus, the structures of most of the minor syringostatins were determined. The minor components, **3** and **7**, gave unassignable signals in mass spectra, and therefore their structures could not be determined.

Recently, Segre *et al.*[9] proposed structures for the syringomycins, which are cyclic lipodepsipeptides. The structures of these latter toxins closely resemble those of the syringostatins reported here. However, significant differences in the amino acid content occur. A discrepancy in the *C*-terminal sequence is also apparent between that reported for syringomycin E and for the syringostatin. In the subsequent paper, a comparative structural study is reported for these toxins including syringotoxin. Additionally, an evaluation of the *C*-terminal sequence of syringomycin is detailed as also discussed recently by Ballio *et al.*[11]

Experimental

FAB Mass Spectrum.—The FAB mass spectra were measured on a JEOL JMS-DX303 mass spectrometer, using glycerol containing hydrogen chloride as matrix and ionized by the impact of the accelerated Xe atom. The high resolution FAB

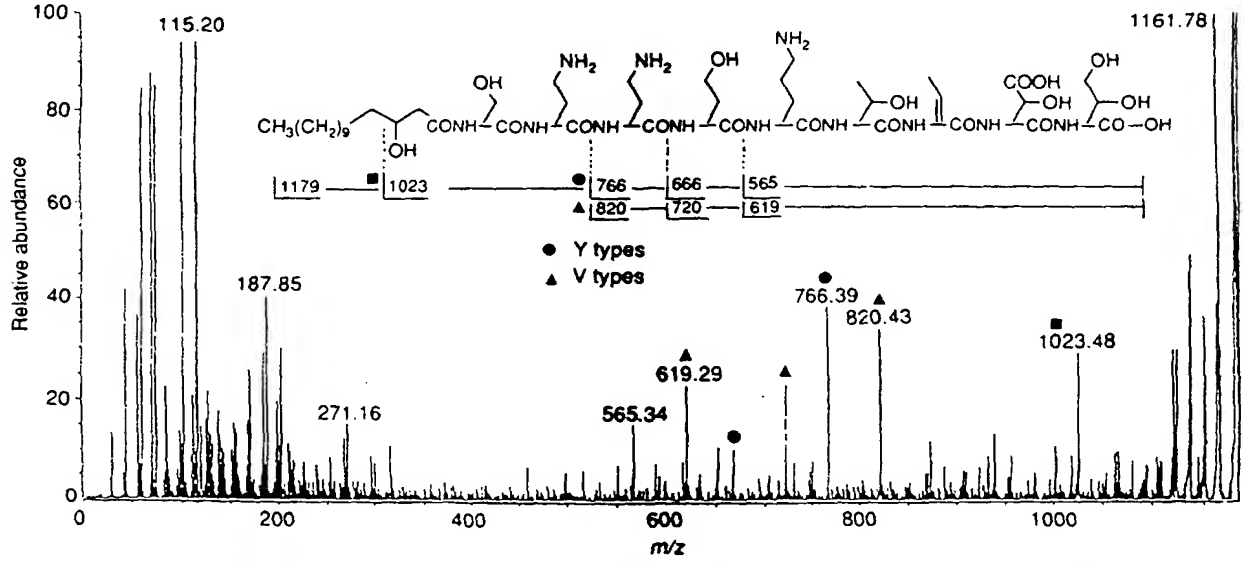


Fig. 5 Mass mass spectrum of SYH-A **10**. Considerable fragmentations are shown in the structure in upper side of the spectrum. Ions marked by closed circles are Y type ions, and closed triangle are V type ions.

mass spectra were measured on a JEOL JMS-SX102 mass spectrometer, using a mixture of glycerol and thioglycerol as matrix and ionized by the impact of the accelerated Xe atom. The exact mass was calculated by comparison with polyethyleneglycol ≈1000 as mass marker. The mass/mass spectra were also measured on the JEOL JMS-SX102/SX102 mass spectrometer.

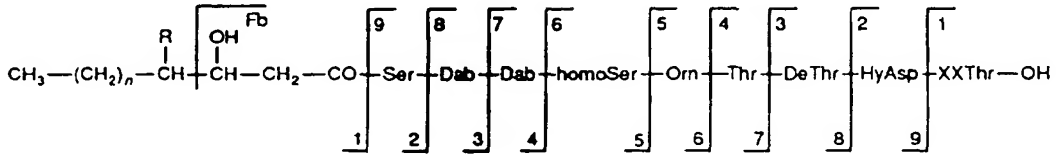
NMR Spectra.—The NMR spectra were measured with Bruker AM-600 and Bruker ACP-300 spectrometers. ^1H NMR spectra were recorded with 32K (AM-600) or 16K (ACP-300) data points, and normalized with residual $CH D_2CN$ (δ_H 2.00) in CD_3CN–H_2O (7:2 v/v), HDO (δ_H 4.80) in D_2O, $CHCl_3$ (δ_H 7.24) in $CDCl_3$ and CHD_2OD (δ_H 3.55) in CD_3OD as internal standards. ^{13}C NMR spectra were recorded with 64K (AM-600) or 32K (ACP-300) data points, and normalized with the signals of CD_3CN (δ_C 1.2), $CDCl_3$ (δ_C 77.0) and CD_3OD (δ_C 49.0) as the internal standards in each solvent and the external standard of dioxane (δ_C 67.8) in D_2O. *J* values are given in Hz throughout.

COSY and DEPT spectra were performed by conventional methods. HOHAHA spectra were measured by the method of Davis and Bax[12] using spin lock pulses (2.5 ms) before and after the 40 times of MLEV17 cycles (mixing time, 80 ms). HMQC spectra and HMBC spectra were measured by the method of Bax and Summer,[13] adopting 3.6 ms as $1/2^1J_{CH}$ and 60 ms as $1/2^nJ_{CH}$ as the developing times. In the HMQC spectra, carbon signals were decoupled by 14 cycles of GARP1 pulses. Phase-sensitive NOESY spectra were obtained by the method of Bodenhauser *et al.*[14] using 800 ms as the mixing time.

Isolation of Indole Acetic Acid.—The activity was assayed by the inhibitory activity on the growth of lettuce seedlings.

P. syringae pv. syringae SY12 was cultured in 5 dm^3 volume Erlenmeyer flasks (2 dm^3 medium in each flask) with Bennett medium (1% glucose, 0.2% polypeptone, 0.1% meat extract and 0.1% yeast extract) at 26.5 °C for 4 d with shaking. The centrifuged supernatant (7000 rpm, 10 °C, 20 min) was adsorbed on a charcoal column (19.7 × 3.6 cm), washed with 20% ethanol (400 cm^3), and the activity was eluted with 20% pyridine (1 dm^3). This eluate was diluted to twice its volume and applied to a DEAE-Sephadex A-25 column (buffered with 10% pyridine), washed with 10% pyridine (200 cm^3), and eluted with a linear gradient of acetic acid (0.02–1.0 mol dm^{-3}, 200 cm^3) and further 1 mol dm^{-3} acetic acid (200 cm^3). The activity was recovered around 1 mol dm^{-3} acetic acid eluate, and the active fractions were concentrated and lyophilysed. The residue was dissolved in small volumes of water and further purified by HPLC with an ODS column (SSC-ODS-H-3201, 200 × 8 mm, Senshu Kagaku Co.), eluted with an acetonitrile gradient (0–20% in 2 min, 20% isocratic for 20 min) in 0.1% TFA (trifluoroacetic acid). The active substance, indole-3-acetic acid was obtained (31.8 mg) from 13 dm^3 of broth; FD-MS; *m/z* 176 [(M + H)$^+$], δ_H(D_2O) 3.72 (s, 2 H), 7.00 (ddd, *J* 1, 7, 8), 7.09 (ddd, *J* 1, 7, 8), 7.16 (s), 7.33 (ddd, *J* 1, 1, 8) and 7.53 (ddd, *J* 1, 1, 8).

Production and Isolation of Syringostatins.—*P. syringae pv. syringae* SY12 was cultured in a medium containing 1% potato extract (Difco), 1.5% glucose and 0.4% casamino acid (Difco), adjusted to pH 7.0. The cultivation was performed without



	R	XXTHr	*n*
9	H	ClThr	9
11	OH	ClThr	9
10 = **15**	H	HyThr	9
12 = **13**	OH	HyThr	9
14	H	ClThr	7
16	OH	ClThr	11

N-terminal fragments in FAB mass spectrum

Compounds	M + H	A2	B2	C2	B3	C3	B4	C4	B5	C5	C6	A7	C7	C8	C9
9 (SYL-A)	1197	286	314	331	414	**431**	514	531	615	632	746	802	847	930	1061
11 (SYL-B)	1213		330			**447**	530	547	631	648	762		863	946	1077
10 (SYH-A)	1179		314		414	**431**	514	531	615	729	746	802	847	930	1061
12 (SYH-B)	1195		330			**447**	530	547	631	648	762	818	863	946	1077
14	1169		286		386	**403**	486	503	587	604	718		819	902	1034
16	1241														

15 = **10** (SYH-A), **13** = **12** (SYH-B)

C-terminal fragments in mass/mass spectrum

Compounds	M − H	Y5	Y6	V6	Y7	V7	V8	Fb
15	1179	565	666	619	766	**720**	820	1023
13	1195	565	666	619	766	**720**	820	1023
14	1169	583	684	637	784	**737**	838	1041
16	1241				784	**738**	838	1041

Fig. 6 Structures and the fragment ions observed in the FAB mass and mass/mass spectrum of syringostatins. Derivatives **13**, **14**, **15** and **16** were obtained from syringostatins D 4, E 5, F 6 and H 8, respectively.

stirring in 500 cm^3 volume Erlenmeyer flasks containing medium (200 cm^3) at 26.5 °C for 7 d. During the process of the isolation, syringostatins were assayed by their antibiotic activity to fungi, *Penicillium chrysogenum*, on potato–dextrose–agar plates. The centrifuged supernatant (8000 rpm, 4 °C, 20 min) of the cultured broth (9 dm^3) was adsorbed on an XAD-7 column (40 × 3.6 cm), which was rinsed preliminarily with 0.2% TFA. After washing with 20% ethanol (1.5 dm^3), the activity was eluted with 80% ethanol (900 cm^3). The concentrated active fractions were dissolved with 0.2% TFA (30 cm^3), stirred for 30 min and centrifuged (16 000 rpm, 4 °C, 15 min), then the supernatant was applied to a Sephadex G-25 column (53 × 2.3 cm) with 0.2% TFA. The column was eluted with 0.2% TFA. The active material was recovered in the fractions of elution volumes 180–230 cm^3. After lyophilization, the active material was chromatographed on a Sephadex LH-20 column (55 × 2.3 cm) with 45% ethanol and 0.5% acetic acid, and fractions (5 cm^3) collected. Fractions 29–36 were concentrated under reduced pressure and lyophiliysed. The residue, dissolved in water, was applied to an HPLC ODS column (SSC–H–3210, 200 × 8 mm, Senshu Kagaku Co.) and eluted with an acetonitrile gradient (25–50% in 20 min) in 0.1% TFA. The main component, syringostatin A (7.4 mg), the mixture of syringostatins B and C (10.0 mg), and the minor syringostatins from D to H (each less than 1 mg) were isolated. The mixture of syringostatins B and C was further resolved by HPLC using another reverse-phase column (SSC–CN–4251–N, 250 × 10 mm, Senshu Kagaku Co.). The yields of syringostatins B and C were 5.5 and 4.5 mg, respectively.

Amino Acid Analysis of Syringostatins.—Syringostatins (*ca.* 120 μg) were hydrolysed in sealed glass tybes with hydrochloric acid (6 mol dm^{-3}; 200 mm^3) at 110 °C for 18 h. The hydrolysate was concentrated under reduced pressure and dissolved in hydrochloric acid (0.02 mol dm^{-3}; 500 mm^3). The sample was analysed for amino acids (Hitachi model-835). The detected known amino acids were allothreonine (1.1 mole, an unknown peak overlapped), serine (0.5 mole), 2,4-diaminobutanoic acid (1.8 mole) and ornithine (0.7 mole) from one mole of syringostatin B **2**.

Analysis of Fatty Acid in Syringostatins.—Hydrolysed syringostatins (200 μg) were dried, dissolved in hydrochloric acid (1 mol dm^{-3}; 200 mm^3), and extracted with ethyl acetate (100 mm^3). After removal of the solvent, the residue was methylated with CH_2N_2 in ether, and the reactant was applied to a GC-MS (JEOL JMS-DX303) with a packed column of OV-17. The column temperature was increased from 80 to 250 °C in 21 min, and the residue ionized by the electron-impact (EI) method. The methylated acid residue of the hydrolysate of syringostatin **A 1** was eluted at 13.8 min, and gave fragment ions at *m/z* 241, 209, 208, 183, 166, 103, 71 and 55. Methylated derivative from syringostatin B **2** was eluted at 14.2 min, and gave ions at *m/z* 225, 169, 143, 130, 115, 111, 98 and 55. The value of *m/z* 241 for syringostatin A was explained by the deletion of an hydroxy group from the methyl ester form, and *m/z* 225 by deletion of an hydroxy group from the γ-lactone form.

Synthesis of 3-Hydroxytetradecanoic Acid.—A solution of anhydrous THF (50 cm^3) was refluxed and vigorously stirred with powdered zinc (3.2 g). Dodecanal (2.0 g, 10.9 mmol) and bromoethylacetate (4.1 g, 24.6 mmol) in anhydrous THF was added dropwise. After refluxing for 1 h, the supernatant was decanted off, and the solvent removed under reduced pressure; further traces of solvent were removed from the reaction mixture at 120–130 °C. Benzene (50 cm^3) was added to the reaction mixture, which was then refluxed for 2 h. After removal of the solvent, 2 mol dm^{-3} HCl was added to the residue and it was extracted with ethyl acetate (3 × 300 cm^3). The residual solid remaining after removal of the solvent was then hydrolysed with 90% ethanol (100 cm^3) and KOH (4.0 g) at room temperature for 40 h. Extraction with ethyl acetate and evaporation of solvent yielded 3-hydroxytetradecanoic acid as a yellow amorphous solid (*ca.* 350 mg), $\delta_C(CDCl_3)$ 14.1, 22.7, 25.4, 29.3, 29.5–29.6 (some signals were overlapped), 31.9, 36.5, 41.1, 66.1 and 177.7, $\delta_H(CDCl_3)$ 0.82 (t, *J* 7), 1.1–1.5 (m), 2.40 (dd, *J* 8, 17), 2.51 (dd, *J* 3, 17). The methylated derivative of this compound showed the same GC-MS peaks and ions as that from the hydrolysate of syringostatin A as shown above.

Base-hydrolysis of Syringostatin A and B.—A solution of syringostatin A (500 μg in 500 mm^3) was combined with Tris-HCl buffer (0.2 mol dm^{-3}; 500 mm^3; pH 8.5), and incubated at 37 °C for 1 h. The reaction was stopped by adding hydrochloric acid (1 mol dm^{-3}; 100 mm^3). The solvent was removed under reduced pressure, and the sample was applied to an ODS column (SSC-ODS-H-3201, 20 × 8 mm, Senshu Kagaku Co.), and the hydrolysed derivative SYL-A was recovered. Similarly the derivative SYH-A was obtained by incubation for 18 h. Syringostatin B and minor compounds of syringostatins were treated in the same way.

Acknowledgements

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Polymyxin Acylase: An Enzyme Causing Intramolecular $N^2 \rightleftarrows N^6$ Acyl Transfer in *N*-Monooctanoyl-L-Lysine

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Polymyxin acylase from *Pseudomonas* sp. M-6-3 can deacylate not only polymyxin antibiotics, but also *N*-fatty acyl-peptides and *N*-fatty acyl-amino acids. We found that this enzyme causes intramolecular $N^2 \rightleftarrows N^6$ acyl transfer in monooctanoyl-L-lysine; when N^2-octanoyl-L-lysine is the substrate, N^6-octanoyl-L-lysine is produced at pH 10.5, but when N^6-octanoyl-L-lysine is the substrate, N^2-octanoyl-L-lysine is produced at pH 8.0. In these reactions, the deacylation proceeded gradually at the final stage and eventually, both N^2-octanoyl-L-lysine and N^6-octanoyl-L-lysine were hydrolyzed to L-lysine and octanoic acid. Furthermore, this enzyme showed intermolecular acyltransferase activity, transferring several *N*-octanoyl-DL-amino acids to *N*-octanoyl-hydroxylamine. This acyltransfer ability of polymyxin acylase offers a new method of enzymic *N*-acylation of compounds containing amino components.

Polymyxin acylase from *Pseudomonas* sp. M-6-3 is an enzyme useful for preparing semisynthetic polymyxins.[1] It can deacylate not only polymyxin antibiotics, but also *N*-fatty acyl-peptides and *N*-fatty acyl-amino acids. The cell-free enzyme solubilized from acetone-dried cell powder has been purified to a homogeneous state and its properties have been clarified with special reference to its broad substrate specificity.[2] In the last stage of the enzymatic deacylation for colistin, HPLC reveals new small peaks with retention times slightly longer than those of colistins A and B. Analysis of these new peaks by the DNP method suggested that an acyl group linked with an α-amino group migrates to a γ-amino group in colistin.[3]

In this study, we examined the acyltransferase activity of polymyxin acylase on N^2-octanoyl-L-lysine as a mimic substrate. This transferase activity has not been found in many known amino acylases. We report here that polymyxin acylase catalyzes not only $N^2 \rightleftarrows N^6$ acyl migration on *N*-monoacyl-L-lysine, but also intermolecular acyl migration from *N*-acyl-DL-amino acids to *N*-acyl-hydroxylamine.

Materials and Methods

Chemicals. N^2-Octanoyl-L-lysine was prepared by removing the benzyloxycarbonyl group from N^2-octanoyl-N^6-benzyloxycarbonyl-L-lysine, which was prepared by the Shotten-Baumann reaction of N^6-benzyloxycarbonyl-L-lysine and octanoyl chloride. N^6-Octanoyl-L-lysine was prepared in the same manner from L-lysine copper chelate and octanoyl chloride. All other chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of polymyxin acylase. Polymyxin acylase was purified from the acetone-dried cell powder of *Pseudomonas* sp. M-6-3 by the procedure described in our preceding paper.[2] The purified enzyme (Type I) was homogeneous by SDS-polyacrylamide gel electrophoresis and the activity for colistin B was 1750 units/mg; one unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of deacyl colistin B per min.

Enzyme assay. Intramolecular acyltransferase activity

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DFP, diisopropylphosphoro fluoridate; PMSF, phenylmethane sulfonylfluoride.

was assayed from the ninhydrin color spot of N^2- or N^6-monooctanoyl-L-lysine on the TLC plate (Method 1). Intermolecular acyltransferase activity was measured using hydroxylamine as the acyl acceptor and *N*-acyl-DL-amino acids as the acyl donor and the rate was assayed by the $FeCl_3$-color reaction of hydroxamic acid (Method 2).

Assay method 1. The reaction mixture, 200 μl, containing 2.5 μmol of N^2-octanoyl-L-lysine and the enzyme solution (10 units) in 100 mM carbonate buffer (pH 10.5), was incubated at 37°C for several hours. For the reverse reaction with N^6-octanoyl-L-lysine, the phosphate buffer (pH 8.0) was used.

A 1 μl portion of the reaction mixture was put on a thin layer plate of silica gel and developed with a solvent system of phenol–water (4 : 1). To detect the product and residual substrate, ninhydrin reagent (80 mg ninhydrin, 1 ml pyridine, and 20 ml ethanol) was sprayed on the plate, which was kept at 100°C for 5 min. The amount of the product was monitored by the ninhydrin color with Chromoscan 200 201 (Joyce-Loebl, England).

Assay method 2. A reaction mixture, 200 μl, containing 25 μmol of *N*-octanoyl-DL-amino acid, 75 μmol hydroxylamine sulfate, and the enzyme solution (30 units) in 100 mM Tris-HCl buffer (pH 8.0) was incubated at 37°C for several hours. To this mixture (50 μl), 5.7 mM $FeCl_3$ solution in 160 mM HCl, 450 μl, was added, and the absorbance at 525 nm was measured.

Results

Production of a ninhydrin-positive compound in the reaction of N^2-octanoyl-L-lysine with polymyxin acylase

The reaction of N^2-octanoyl-L-lysine and polymyxin acylase yielded a ninhydrin-positive compound which appeared at a position on the thin-layer plate different from that of lysine. With time, the lysine gradually appeared. This compound was found to be N^6-octanoyl-L-lysine by comparison of its thin-layer chromatographic and high-voltage electrophoretic data with those of an authentic sample.

Effects of pH on intramolecular acyltransferase activity

The migration of the octanoyl group from N^2 to N^6 on L-lysine was examined. In the reaction mixture with N^2-octanoyl-L-lysine and polymyxin acylase, the optimal pH for N^6-octanoyl-L-lysine formation (*N*-acyltransferase activity) was 10.5, and that for L-lysine formation (deacylase activity) was 8.5 (Fig. 1).

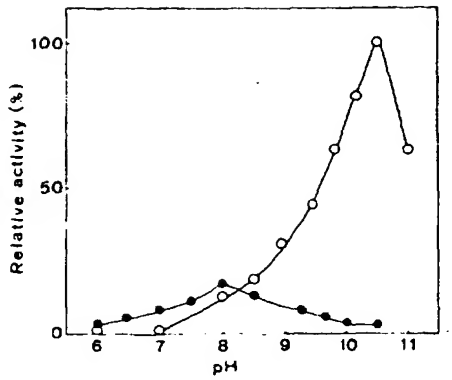

Fig. 1. Effects of pH on Intramolecular Acyltransferase Activity in *N*-Monooctanoyl-L-lysine.

—○—, $N^2 \rightarrow N^6$ acyltransferase activity; —●—, $N^6 \rightarrow N^2$ acyltransferase activity.

The reaction mixture, 200 μl, containing 2.5 μmol of N^2-octanoyl-L-lysine (or N^6-octanoyl-L-lysine) and 10 units of enzyme in the following buffers (final concentration of 100 mM): citrate–phosphate (pH 6.0), sodium phosphate (pH 6.5 ~ 8.5), Tris HCl (pH 7.5 ~ 9.0), and sodium carbonate (pH 9.5 ~ 11.0), was incubated for 1 hr at 37°C. The relative activity is expressed as the percentage of the maximum activity (2700 nmol/min/mg protein) attained under these experimental conditions.

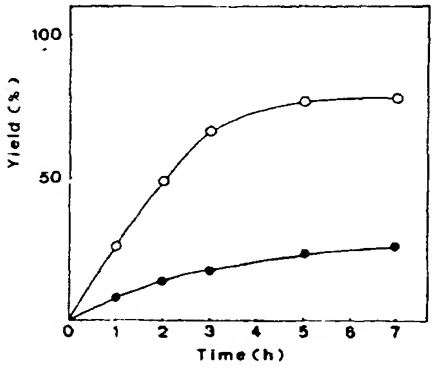

Fig. 2. Course of N^6-Octanoyl-L-lysine Formation from N^2-Octanoyl-L-lysine.

—○—, N^6-octanoyl-L-lysine; —●- -, L-lysine.

The reaction mixture, 500 μl, containing 10 μmol of N^2-octanoyl-L-lysine and 30 units of enzyme in 100 mM carbonate buffer (pH 10.5), was incubated at 37°C.

At pH 10.5, the deacylase activity was much weaker than the *N*-acyltransferase activity. The courses of the *N*-acyltransfer and deacylation reactions at pH 10.5 showed a predom-

inance of N^6-octanoyl-L-lysine formation at the initial stage and a gradual appearance of L-lysine (Fig. 2). Eventually, the N^6-octanoyl-L-lysine that formed was hydrolyzed to L-lysine and octanoic acid. The apparent V_{max} and *Km* values for N^2-octanoyl-L-lysine were 2700 nmol/min/mg and 1.75 mM, respectively. For the reverse reaction ($N^6 \rightarrow N^2$ octanoyl migration on L-lysine), the optimal pH was lower, pH 8.0. The course of the enzyme reaction showed that N^2-octanoyl-L-lysine formation from N^6-octanoyl-L-lysine was predominant and L-lysine gradually appeared as found in the $N^2 \rightarrow N^6$ migration reaction. However, the V_{max} value for N^6-octanoyl-L-lysine (450 nmol/min/mg) was smaller than that for N^2-octanoyl-L-lysine.

Effects of metal ions and chemical reagents on intramolecular acyltransferase activity

The acyltransferase activity was measured from the migration of the octanoyl group from N^2 to N^6 on L-lysine. The enzyme activity was markedly inhibited by Hg^{2+} and Ag^{+}, inhibited (50%) by Cu^{2+}, but slightly enhanced by Co^{2+} and Mg^{2+} at the concentration of 1 mM. Inhibition by other agents at 1 mM concentration was as follows: metal ion chelating agents (EDTA, EGTA, *o*-phenanthroline and 8-hydroxyquinoline) 5 ~ 10% inhibition; thiol-blocking reagents, 20 ~ 30% inhibition, and an oxidizing agent, *N*-bromosuccinimide, complete inhibition.

Tolerance for organic solvents on the intramolecular acyltransferase activity

The enzyme showed tolerance for several organic solvents, acetone, ethanol, ethylene glycol, and acetonitrile. Among them, ethylene glycol was best tolerated. Interestingly, addition of 20 ~ 30% ethylene glycol enhanced, and did not inhibit, the enzyme activity, and even in 50% ethylene glycol, half of the activity remained. All organic solvents examined increased the enzyme activity 10 ~ 30% when added as 10% solutions (Fig. 3).

Intermolecular acyltransferase activity of polymyxin acylase

The migration of the octanoyl group on *N*-octanoyl-DL-amino acids from DL-amino acid to hydroxylamine was examined. The reaction mixture with *N*-octanoyl-DL-glutamic acid, hydroxylamine, and polymyxin acylase gave *N*-octanoyl-hydroxylamine and the optimal pH was 8.5. The apparent V_{max} and *Km* values were 800 nmol/min/mg and 36 mM for hy-

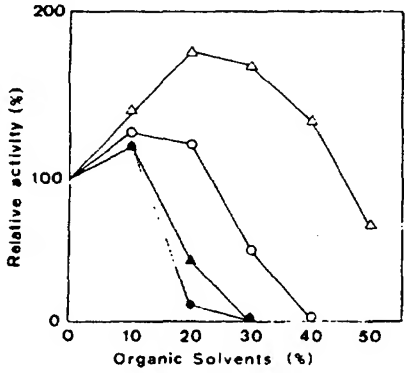

Fig. 3. Effects of Organic Solvents on the Intramolecular Acyltransferase Activity of Polymyxin Acylase (Type I).

The enzyme activity was measured in the presence of various concentrations of organic solvents.
—○—, acetone; — —, acetonitrile; —▲—, ethanol; △—, ethylene glycol.

Table I. Substrate Specificity of Acyltransfer of *N*-Octanoyl-DL-amino Acids to *N*-Octanoyl-hydroxylamine by Polymyxin Acylase (Type I)

Acyl donors	Relative activity (%)
N-Octanoyl-DL-glutamic acid	100
N-Octanoyl-glycine	100
N-Octanoyl-DL-valine	90
N-Octanoyl-DL-aspartic acid	90
N-Octanoyl-DL-serine	75
N-Octanoyl-DL-threonine	75
N-Octanoyl-DL-alanine	75
N-Octanoyl-DL-methionine	60
N-Octanoyl-DL-leucine	40
N-Octanoyl-DL-phenylalanine	10

The reaction was done at 37°C for 1 hr by assay method 2 (pH 8.0) using 30 units of enzyme. The formation of *N*-octanoyl-hydroxylamine, corresponding to 800 nmol/min/mg protein, was taken as 100%.

droxylamine, respectively. The other *N*-octanoyl-DL-amino acids were also effective as acyl donors, and the substrate specificity for amino acid residues on intermolecular acyltransferase activity was slightly different from that of the amino acid acylase activity of the same enzyme. As shown in Table I, *N*-octanoyl derivatives of some amino acids (Gly, Val, Asp, and Glu) were useful as the acyl donor. Neither metal ion chelating agents nor thiol-blocking reagents, except for *p*-chloromercuribenzoate, affected the intermolecular acyltransferase activity at 1 mM. *N*-Bromosuccinimide inhibited the activity in a similar manner to its inhibition of intramolecular acyltransferase activity.

Discussion

Previously, we found that polymyxin acylase from *Pseudomonas* sp. M-6-3 displays $N^2 \rightarrow N^4$ acyl migration activity on 2,4-diaminobutyrate in the polymyxin molecule.[3] To clarify the mechanism of this activity, N^2-octanoyl-L-lysine was used as a mimic substrate, because polymyxin acylase has a high affinity for long chain fatty acyl groups and lysine is a typical basic amino acid. The optimal pH for the acyl migration from N^2-octanoyl-L-lysine to N^6-octanoyl-L-lysine was 10.5, which is equal to the pK value of the ε-amino group of lysine. This means that the un-protonated form of the ε-amino group is required for the acceptance of the acyl group and a much higher concentration of hydroxy ion may disturb this activity by a conformational change of the enzyme. In the reverse reaction (from N^6-octanoyl-L-lysine to N^2-octanoyl-L-lysine), the optimal pH was 8.0, which was also approximately equal to the pK value of the α-amino group of lysine. These results suggest that the optimal pH of N,N-acyltransferase is approximately equal to the pK value of the amino group that will accept an acyl group. The courses of $N^2 \rightarrow N^6$ and $N^6 \rightarrow N^2$ acyl migration on lysine by polymyxin acylase indicated that the acyltransfer reaction occurs first and is followed by deacylation (Fig. 4). A small amount of direct liberation of the acyl group from the N^2-octanoyl-L-lysine or N^6-octanoyl-L-lysine was also recorded. Our results showed that the migration by polymyxin acylase from N^2-octanoyl-L-lysine to N^6-octanoyl-L-lysine occurred much more easily than that from N^6-octanoyl-L-lysine to N^2-octanoyl-L-lysine, which offers evidence in support of the acyltransfer occurring before the deacylation in the polymyxin molecule.

The acyltransferase activity showed tolerance to several organic solvents as seen for the deacylase activity on polymyxin. The tolerance observed for the acyltransferase activity was a little higher than that for the deacylase activity. For example, addition of 20% ethylene glycol led to 80% activation rather than inhibition of the acyltransferase activity. This

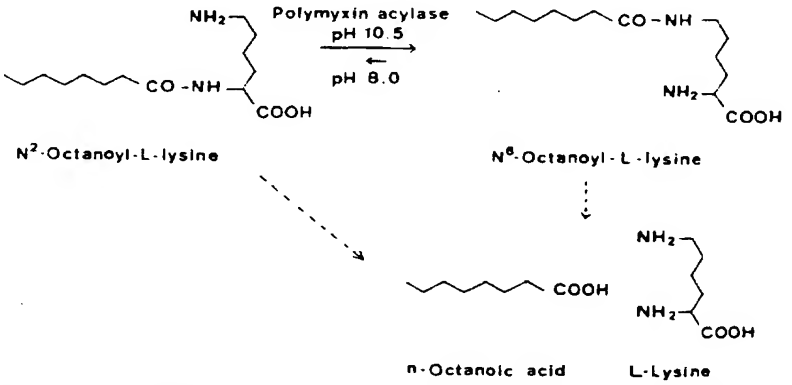

Fig. 4. Scheme of Intramolecular *N*-Acyltransfer of N^2-Octanoyl-L-lysine to N^6-Octanoyl-L-lysine by Polymyxin Acylase.

may have been due to repression of protonation on the amino group, which serves as the acceptor, by the addition of organic solvents. The optical specificity of the enzyme for lysine was not exact; it acted on both *N*-octanoyl-L-lysine and *N*-octanoyl-D-lysine, but the activity for the L-form was 10 times higher than that for the D-form. The substrate specificity for acyl groups other than the octanoyl group has not yet been studied, but the deacylase activity for many fatty acyl groups in *N*-acyl-methionine has been reported.[2]

This enzyme showed not only intramolecular acyltransferase activity, but also intermolecular acyltransferase activity from *N*-octanoyl-DL-amino acids to *N*-octanoyl-hydroxylamine. However, these V_{max} values were lower than that in the intramolecular acyltransferase activity for the acyltransfer of N^2-octanoyl-L-lysine to N^6-octanoyl-L-lysine. The *Km* value for hydroxylamine was much higher than the usual values. Generally, the acyl donor of acyltransferase is acyl-S-CoA, but we found here the presence of *N*,*N*-acyltransferase as the donor for *N*-acyl-amino acids. However, there is little possibility of this enzyme displaying this activity in living cells because of its high *Km* value.

Intramolecular acyltransferase activity displayed by polymyxin acylase was inhibited by Hg^{2+} and Ag^{+}, but was affected little by metal chelators and various thiol-blocking reagents, other than *p*-chloromercuribenzoate. These properties are very similar to those of its deacylation activity. Both reactions, deacylation and transacylation, may occur at the same active site of the enzyme. None of the known aminoacylases that we examined showed this acyltransferase activity. This means that the enzymic reaction mechanism of polymyxin acylase is different from those of usual aminoacylases. Similar enzymes, penicillin acylases, produced from *E. coli*,[4] *Bacillus megaterium*,[5] or *Xanthomonas citri*[6] are also distinguishable from polymyxin acylase, because they are serine enzymes, inhibited by DFP or PMSF.

In sum, polymyxin acylase shows both intra- and intermolecular acyltransferase activities. It can cause *N*,*N*-acylmigration of the *N*-monoacyl derivatives of basic peptides, which shows promise for use in preparing the acyl isomers of these peptides. It can also be a very useful deacylating reagent for many *N*-acyl-peptides. This intermolecular acyl transfer ability of polymyxin acylase shows promise for the development of a new method of enzymic *N*-acylation of compounds containing amino components.

Acknowledgment. The authors thank Professor Kyozo Hayashi, Gifu Pharmaceutical University, for his helpful advice.

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Polymyxin Acylase: Purification and Characterization, with Special Reference to Broad Substrate Specificity

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Polymyxin acylase, which produces deacylated polymyxins by hydrolyzing only the fattyacyl groups of polymyxin antibiotics without affecting the peptide moiety, was purified from acetone-dried cell powder of *Pseudomonas* sp. M-6-3. The cell-free enzyme, solubilized by Triton X-100, was further purified on successive DEAE-cellulose, hydroxyapatite, and Sephacryl S-300 columns to a homogeneous state. This purified enzyme (Type I) had a single band with an MW of 62,000 in SDS-polyacrylamide gel electrophoresis. Gel filtration on a calibrated Sephacryl S-300 column also gave an estimated MW of 62,000. The isoelectric point of the enzyme was 5.7. Enzyme activity was optimal at pH 9.0 for colistin A (polymyxin E_1) and was stable up to 50°C for 24 hr. The observed V_{max} and Km values were 1750 nmol/min/mg and 3.85 mM, respectively. This enzyme was almost not affected by metal chelators and various thiol-enzyme inhibitors (other than *p*-chloromercuribenzoate), and showed high tolerance for organic modifiers; for example, half of the activity remained in 50% ethylene glycol buffer. This enzyme deacylated not only polymyxins but also various *N*-fattyacyl compounds (peptides and amino acids). Among several fattyacyl groups (C_2–C_{16}) of *N*-acyl DL-methionines, the caprinoyl (C_{10}) group was most easily liberated, and the benzyloxycarbonyl (Z) group was also slightly susceptible. When the enzyme was solubilized in 0.2 M KCl-containing buffer by Triton X-100, the enzyme (Type II) showed a slightly different substrate specificity and an increased activity for some Z-derivatives. With this enzyme, it is possible to remove the Z group from several Z-peptides under mild conditions.

The acyl peptide antibiotics, polymyxin B and colistin (polymyxin E), have been used clinically against Gram-negative bacteria. However, little has been done to improve their therapeutic value by creating semisynthetic polymyxins *via* replacement of the acyl group. Previously, we found a strain, *Pseudomonas* sp. M-6-3,[1] which produces polymyxin acylase, a new enzyme for preparing the starting material for semisynthetic polymyxins. We showed that the cell-bound enzyme acts on polymyxin to produce deacylated polymyxin and fatty acid(s). Furthermore, the cell-bound enzyme can produce deacylated peptides from the acyl peptide antibiotics, cerexin (by Shoji & Kato)[2], brevistin (by Shoji & Kato),[3] echinocandin B (by Pache *et al.*),[4] and neopeptin (by Ubukata *et al.*).[5] This paper is concerned with the solubilization, purification, and characterization of polymyxin acylase from *Pseudomanas* sp. M-6-3. Since polymyxin acylase is unique in having a broader substrate specificity than several other known aminoacylases, it should be useful as a deacylating reagent for many *N*-acyl compounds.

Materials and Methods

Strain and growth conditions. Pseudomonas sp. M-6-3 strain was isolated in our laboratory from a soil sample from Nishinomiya, Japan.[1] The organisms were grown aerobically in medium containing 10 g of sodium citrate, 0.1 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeCl_3 \cdot 6H_2O$, and 0.01 g

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid; TPCK, *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone; TLCK, *p*-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride; DFP, diisopropylphosphoro fluoridate; Z, *N*-benzyloxycarbonyl.

of $CaCl_2 \cdot 2H_2O$ per liter of water at pH 7.5 which was shaken at 28°C for 72 hr. The bacterial mass was harvested, and the acetone-dried cell powder was prepared by the usual method, giving a yield of 0.9 g/l.

Chemicals. Colistin sulfate was kindly supplied by the Banyu Pharmaceutical Co. (Tokyo, Japan). Colistin A was prepared from a colistin complex by HPLC and *N*-fattyacyl methionines were prepared by the water-soluble active ester method[5] from the appropriate fattyacyl ester of *p*-dimethylsulfoniophenol methylsulfate and methionine in our laboratory. *N*-Benzyloxycarbonyl peptides were the generous gifts of Dr. M. Fujino, Takeda Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan).

Enzyme assay. Polymyxin acylase was assayed from the UV-absorption peak of deacylated peptide on HPLC (method 1). Acylase activity for acyl amino acids was assayed by the ninhydrin color of the liberated amino acid (method 2). Urethane-hydrolase activity for *N*-benzyloxycarbonyl derivatives was assayed from the UV-absorption of the liberated benzyl alcohol on HPLC (method 3).

Assay method 1. The reaction mixture, 200 μl, containing 2 μmol of colistin sulfate and the enzyme solution (100 μl) in 50 mM Tris buffer (pH 9.0), was incubated at 37°C for several hours. After each hour, 100 μl of 1 N H_2SO_4 was added and the mixture was filtered using a 0.45-μm filter (TOSOH, Tokyo, Japan). A 10-μl portion of the filtrate was injected into the HPLC [column, Ultrasphere ODS 5 μm (4.6 × 250 mm) (Beckman, U.S.A.); mobile phase, MeOH : 0.01 N H_2SO_4 = 1 : 1; detector, UV (210 nm)]. The amount of deacyl colistin was calculated from the height of the peak on the chromatogram. One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of deacyl colistin per min.

Assay method 2. The reaction mixture, 200 μl, containing 10 μmol of acyl amino acid (usually *N*-capryloyl-DL-methionine) sodium salt, and the enzyme solution (100 μl) in 50 mM Tris buffer (pH 8.0), was incubated at 37°C for several hours. The reaction mixture was treated as in method 1 and the amount of liberated amino acid in 100 μl of the filtrate was measured colorimetrically by the ninhydrin method.

Assay method 3. The reaction mixture, 200 μl, containing 1 μmol *N*-benzyloxycarbonyl peptide and the enzyme solution (100 μl) in 50 mM Tris buffer (pH 8.0) was treated as in method 1. Next, 10 μl of the enzyme reaction solution was injected into the HPLC [column, Shodex OHpak B-804 (8.0 × 500 mm) (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, water; detector, UV (210 nm)]. The amount of the liberated benzyl alcohol was calculated from the height of the peak on the chromatogram.

Solubilization of the enzyme from acetone-dried cell powder. The powder prepared above (100 mg) was used throughout the following treatments.

a) Sonication: The powder was suspended in 10 ml of 100 mM phosphate buffer (pH 7.2), and disrupted in the presence of a few drops of toluene with a 10 kHz ultrasonic oscillator for 30 min. The cellular debris was removed by centrifugation at 10,000 × *g* for 30 min.

b) EDTA-Lysozyme: The powder was suspended in 10 ml of 5 mM phosphate buffer (pH 6.8), containing 50 μmol EDTA and 50 μmol 2-mercaptoethanol, and then digested by the addition of egg white lysozyme (100 μg/ml) at 30°C for 4 hr. The broth was centrifuged to remove cellular debris.

c) Colistin: The powder was suspended in 10 ml of 10 mM phosphate buffer (pH 7.2), containing 1 mg of colistin sulfate and 1 μmol 2-mercaptoethanol. The mixture was stirred at 28°C for 15 hr, and centrifuged.

d) Triton X-100: The powder was suspended in 10 ml of 10 mM phosphate buffer (pH 7.2), containing 10 mg of Triton X-100 and 1 μmol 2-mercaptoethanol. The mixture was stirred at 28°C for 15 hr and then centrifuged.

Protein measurement. Protein was measured by the method of Lowry *et al.*,[7] with bovine serum albumin (Sigma Chemical Co., U.S.A.) as the standard. In all chromatographic procedures the protein concentration was measured by the absorbance at 280 nm.

Purification of enzyme. One gram of acetone-dried cell powder of *Pseudomonas* sp. M-6-3 was suspended in 30 ml of 10 mM phosphate buffer (pH 8.0) containing 100 mg of Triton X-100. The suspension was stirred at 20°C for 15 hr and then centrifuged at 10,000 × *g* for 30 min. The supernatant was put on a DEAE-cellulose (DE53; Whatman, U.K.) column (1.5 × 21 cm; equilibrated with 10 mM phosphate buffer at pH 8.0), eluted first with the same buffer, and then with a linear gradient from 0 to 500 mM KCl in the equilibration buffer. The active fractions were effectively eluted in the first step. Because the capacity of the column was small, this procedure was repeated several times and the active fractions were pooled. The enzyme from the above step was put on a hydroxyapatite (Nippon Chemical Co., Japan) column (1.5 × 14 cm; equilibrated with 10 mM phosphate buffer at pH 8.0), eluted first with the same buffer, and then with a linear gradient from 10 to 500 mM of phosphate (pH 8.0). The active fractions emerged at two peaks: Fr. I, which showed the most enzyme activity, was re-chromatographed on a hydroxyapatite column as above. The resulting active fractions were combined and then concentrated with a Millipore Immersible-CX Ultrafilter. This concentrated enzyme was put on a Sephacryl S-300 (Pharmacia Fine Chemicals Co., Sweden) column (1.5 × 82 cm; equilibrated with 10 mM phosphate buffer at pH 8.0). The lower molecular weight fraction was re-chromatographed on a Sephacryl S-300 column. The enzyme, which showed a single peak, was eluted and all samples were pooled.

Enzyme homogeneity. The purified enzyme was dialyzed against distilled water, and the Triton X-100 in the enzyme solution was removed with chloroform.[8] SDS-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn,[9] with a 7.5% polyacrylamide gel and phosphate buffer (pH 7.0). Electrophoresis was done at 8 mA per column and gels were stained with Coomassie brilliant blue R-250.

Isoelectric point. Isoelectric-focusing electrophoresis was done by the method of Vesterberg and Svensson[10] using an isoelectrofocusing column (2.5 × 30 cm) (LKB-Produkter. AB, Sweden) and 1% carrier Ampholine (pH 3 ~ 10); electrophoresis was done at 4 C and 700 V for 45 hr.

Measurement of molecular weight. The MW of the purified enzyme was measured by gel filtration on a Sephacryl S-300 column (1.5 × 80 cm) by the method of Andrews,[11] using a standard protein kit (Pharmacia Fine Chemicals Co., Sweden). SDS-polyacrylamide gel (7.5%) electrophoresis as described above was also used to measure the MW of the enzyme.

Results

Purification of polymyxin acylase from Pseudomonas sp. M-6-3

Because polymyxin acylase is bound to the cell envelope, one of the critical steps in purifying it is solubilizing the enzyme from the cell. As shown in Table I, Triton X-100 treatment gave the best yield of the soluble enzyme, although colistin, a substrate of polymyxin acylase, solubilized the enzyme to a lesser extent. The cell free extract solubilized by Triton X-100 was purified by three-stage chromatography; the results are summarized in Table II. Figure 1A shows the chromatographic profile of the enzyme on a DEAE-cellulose column with a linear gradient of KCl. Using colistin as substrate, 90% of the deacylase activity was detected in the passed solution and 10% in the 500 mM-KCl eluate. The enzyme was further purified twice by hydroxyapatite column chromatography (Fig. 1B) and then twice by Sephacryl S-300 column chromatography. When enzyme homogeneity was assessed by SDS-polyacrylamide gel electrophoresis, a single band of protein was observed (Fig. 2). The properties of the purified enzyme were as follows:

Table I. The Enzyme Activity Solubilized from Acetone-dried Cell Powder by Various Treatments

Methods	Treatment time (hr)	Solubilized enzyme activity (units/100 mg powder)
Sonic	0.5	4,000
EDTA-Lyzozyme	4	900
Colistin	15	3,600
Triton X-100	15	7,800

Table II. Purification of Polymyxin Acylase from *Pseudomonas* sp. M-6-3

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	137.0	47,300	340	100
DEAE-cellulose	64.4	41,900	650	89
1st Hydroxyapatite	23.1	31,200	1,350	66
2nd Hydroxyapatite	19.9	31,000	1,560	66
1st Sephacryl S-300	9.5	16,400	1,720	35
2nd Sephacryl S-300	6.4	11,200	1,750	24

Stability

The enzyme was stable for only 5 weeks at 4 C, but the presence of 0.1 M ammonium sulfate or 1% bovine serum albumin increase the period of stability to 4 months. Although the enzyme was stable up to 50 C for 24 hr, there was a marked loss of activity after storing at −40 C, and also after incubating at over 60 C for 1 hr.

Optimal pH and temperature

The optimal pH was 9.0 for colistin A and the optimal temperatures of the enzyme was about 45 ~ 50 C (although *Pseudomonas* sp. M-6-3 itself cannot tolerate temperatures over 35 C). The observed V_{max} and Km values were 1750 nmol/min/mg and 3.85 mM, respectively. For *N*-capryloyl-DL-methionine, these values were 2470 nmol/min/mg and 3.0 mM.

Measurement of MW and isoelectric point

The MW of the purified enzyme was esti-

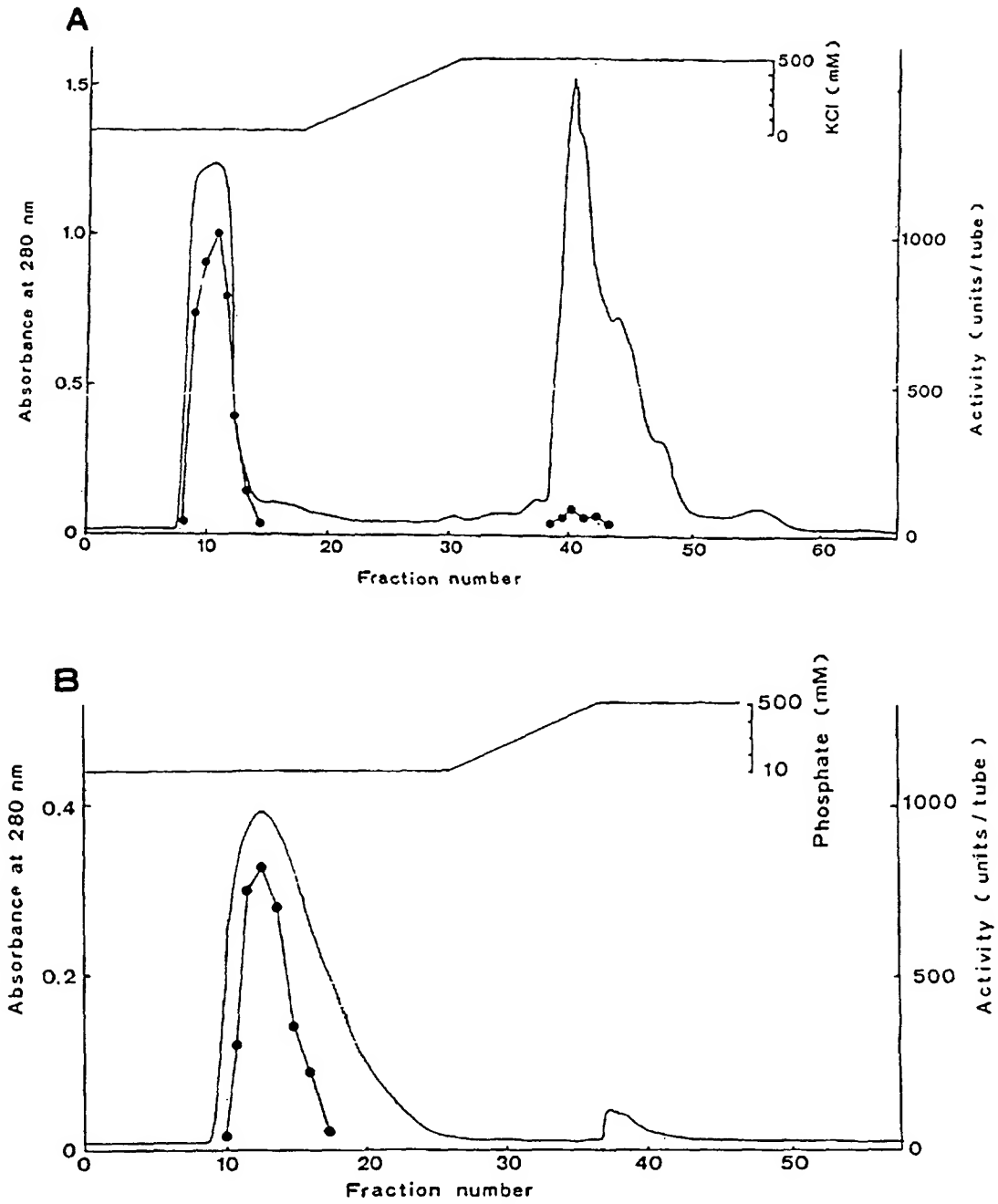

Fig. 1. Column Chromatography of the Polymyxin Acylase on DEAE-Cellulose (A) and Hydroxyapatite (B).

Eluent: (A) 10 mM phosphate buffer (pH 8.0) and then with a linear gradient from 0 to 500 mM KCl in the buffer; (B) 10 mM phosphate buffer (pH 8.0) and then with a linear gradient from 10 to 500 mM of phosphate buffer; flow rate, 48 ml/hr; fraction, 3 ml/tube; ——, absorbance at 280 nm; - - -, enzyme activity.

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Purified Polymyxin Acylase (Type I).

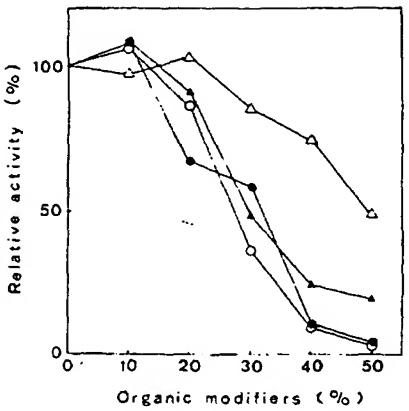

Fig. 3. Effects of Organic Modifiers on the Activity of Polymyxin Acylase (Type I).

The enzyme activity was measured in the presence of various concentrations of organic modifiers by assay method 2. ○—○, methanol; ●- ●, ethanol; △—△, ethylene glycol; ▲- -▲, *N,N'*-dimethylformamide.

mated to be 62,000 by gel filtration chromatography on Sephacryl S-300, but occasionally a high molecular weight peak that had the same enzyme activity emerged. The MW calculated by SDS-polyacrylamide gel electrophoresis was 62,000, which agrees well with gel filtration method. The isoelectric point was 5.7, as measured by isoelectric-focusing electrophoresis.

Effects of metal ions, chemical reagents, and inhibitors

The enzyme activity was markedly inhibited by Hg^{2+} and Ag^{+}, although the other cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ba^{2+}, and Pb^{2+}; 1 mM) had little effect. Metal-ion chelating agents (EDTA, EGTA, *o*-phenanthroline, 8-hydroxyquinoline) did not inhibit the enzyme over the concentration range 0.1 ~ 1.0 mM, but 20 mM EGTA inhibited about 50% of the activity. Neither thiol-protease inhibitors (other than *p*-chloromercuribenzoate) nor serine protease inhibitor (DFP) affected the enzyme activity. TPCK and TLCK did not inhibit. The enzyme was inhibited by two oxidizing agents, *N*-bromosuccinimide and potassium ferricyanate.

Table III. Substrate Specificity of Polymyxin Acylase (Type I)

Substrate	Relative activity (%)
Acetyl-(C_2)-DL-methionine	3.0
Butyroyl-(C_4)-DL-methionine	2.3
Caproyl-(C_6)-DL-methionine	64
Capryloyl-(C_8)-DL-methionine	100
Capryloyl-(C_8)-DL-aspartic acid	122
Capryloyl-(C_8)-DL-glutamic acid	131
Capryloyl-(C_8)-DL-glycine	116
Capryloyl-(C_8)-DL-alanine	111
Capryloyl-(C_8)-DL-valine	105
Capryloyl-(C_8)-DL-leucine	83
Capryloyl-(C_8)-DL-serine	98
Capryloyl-(C_8)-DL-threonine	95
Capryloyl-(C_8)-DL-phenylalanine	83
Caprinoyl-(C_{10})-DL-methionine	107
Lauroyl-(C_{12})-DL-methionine	100
Myristoyl-(C_{14})-DL-methionine	85
Palmitoyl-(C_{16})-DL-methionine	56
Benzoyl-DL-methionine	0.36
Benzyloxycarbonyl-DL-methionine	0.25

Tolerance for organic modifiers

The enzyme showed tolerance for several

Table IV. DIFFERENCE OF SUBSTRATE SPECIFICITY OF POLYMYXIN ACYLASES (TYPES I AND II) ON SEVERAL *N*-BENZYLOXYCARBONYL AMINO ACIDS

Substrate	Relative activity (%)	
	Type I	Type II
Capryloyl-DL-methionine	100	62
Z-DL-Glutamic acid	5.7	18
Z-DL-Aspartic acid	2.9	8
Z-DL-Methionine	0.25	0.3
Z-DL-Glycine	0.2	0.6
Z-DL-Alanine	0.2	0.6
Z-DL-Tyrosine	0.1	0.2

Table V. REMOVAL OF *N*-BENZYLOXYCARBONYL GROUP USING POLYMYXIN ACYLASE (TYPE II)

The reaction mixture (containing 170 units of enzyme) was incubated at 37°C for 48 hr and the removal of the Z group was confirmed by assay method 3.

Substrate	
Z-Glu(OBu^t)-Ser-Thr-Leu	+
Z-Ser-Glu(OBu^t)-Lys(Boc)-Ser-Glu-Thr-Pro	+
Z-Gly-Gly	+
Z-Gly-Gly-NH_2	+
Z-Gly-Phe	+
Z-Ala-Thr-NH_2	+
Z-Pro-Ser-Asp(OBu^t)-Thr-Pro-Ile-Leu-Pro-Gln-OBu^t	−
Z-Pro-Glu(OBu^t)-Ile	−

+, completely removed; −, not removed.

organic modifiers shch as methanol, ethanol, ethylene glycol, and dimethyl formamide (Fig. 3). Even in 50% ethylene glycol buffer, half of the activity remained.

Substrate specificity

Interestingly, this enzyme had a broader substrate specificity than was expected; for example, it hydrolyzed all of the *N*-acyl D- and L-amino acids that were studied. In the case of *N*-acyl methionines, the enzyme was particularly active for long chain fattyacyl groups, especially capryloyl (C_8), caprinoyl (C_{10}), and lauroyl (C_{12}) groups, but also hydrolyzed *N*-acetyl and *N*-benzoyl DL-methionine slightly (Table III). The activity of the enzyme was equivalent for all *N*-capryloyl amino acids, regardless of the amino acid residues (Table III).

On the other hand, when the enzyme was solubilized by the Triton X-100 method in 0.2 M KCl-containing buffer and was purified in the same way, a single homogeneous protein could not be obtained, and this only partially purified preparation showed a slightly different specificity pattern. The relative activity increased for Z-amino acids (Table IV). We called this enzyme preparation Type II to distinguish it from the enzyme solubilized without KCl (Type I). Despite its rather weak activity, a large amount of the Type II enzyme was able to split the Z group of several Z-peptides much more readily than the Type I could. The results are summarized in Table V.

Discussion

Since Schmiedberg[12] observed enzymatic hydrolysis of *N*-benzoyl-glycine in the late 19th century, many kinds of enzymes which are able to hydrolyze *N*-acyl amino acids have been reported. In the early stages, interest centered around the optical resolution of racemic amino acids, as exemplified by the work of Greenstein *et al.* with hog kidney acylases,[13] Kameda *et al.* with bacterial acylases,[14] and Chibata *et al.* with fungus acylases.[15] In the late fifties, interest shifted to penicillin-deacylating enzyme(s) and their use in preparing semisynthetic penicillin. Rolinson *et al.*[16] and Kameda *et al.*[17] published some of the earliest reports; this was followed by a spate of related reports. An earlier paper of ours[1] reported the isolation of a polymyxin-deacylating enzyme (polymyxin acylase) in the cell-bound form and its use in preparing deacyl colistin. The cell-bound enzyme deacylated not only polymyxins but also various *N*-fatty acyl peptides and *N*-acyl amino acids. The substrate specificity of the cell-bound enzyme distinguishes it from other aminoacylase, which usually can hydrolyze *N*-acyl amino acids but not *N*-acyl peptides. In these experiments, we solubilized and purified this new enzyme. We found that the substrate, colistin, is able

to solubilize the cell-bound enzyme; this is consistent with Teuber's observation that polymyxin–lysozyme caused spheroplast formation in *Salmonella*.[18] A suspension of the acetone-dried cell powder of *Pseudomonas* sp. M-6-3 was effective as the enzyme source because of the detergent-activity of colistin on the cell-bound enzyme. The solubilization ability of Triton X-100 was even higher than that of colistin. The solubilizing data in Table I suggest that this enzyme is periplasmic.

After purifying the solubilized enzyme by three-stage column chromatography, we identified it as a single protein by SDS-polyacrylamide gel electrophoresis. The enzyme had a molecular weight of approximately 62,000 and in the presence of Triton X-100, it was liable to polymerize. The properties (substrate specificity, pH-stability, heat tolerance, *etc.*) were very similar to those of the cell-bound enzyme, although the optimum pH of the purified enzyme was slightly higher. Like *N*-long chain acyl aminoacylase from *Pseudomonas diminuta*,[19] chelators such as EDTA and 8-hydroxyquinoline showed no effects on enzyme activity. This is consistent with the inertness of metal ions toward enzyme activity. Also serine protease inhibitors were not active. The activity of polymyxin acylase was not affected by various sulfhydryl agents other than *p*-chloromercuribenzoate.

The most interesting property of this enzyme(s) was its broad substrate specificity. Especially, the specificity for the amino moiety is not exact; for example the activities for *N*-capryloyl amino acids were indistinguishable, regardless of the amino acid residues; it acted not only on the L-form but also on the D-form, and L-form was more easily hydrolyzed. Furthermore, even *N*-capryloyl derivatives having an amino moiety, such as glucosamine, were hydrolyzed by this enzyme.[20] This polymyxin acylase is particularly active on long chain fattyacyl groups, like that of amino acylase from *Mycobacterium phlei*[21] or *Pseudomonas dismuta*.[17] Interestingly, different conditions in enzyme solubilization gave different specificity patterns for the solubilized enzyme. "Type I enzyme," solubilized without KCl, was more active towards *N*-fattyacyl amino acids; while "Type II enzyme," solubilized with KCl, was more active for Z-amino acids. The mechanism of the formation of Type II is not clear. At present, there are two possible explanations of these results. The first would postulate the existence of both types of enzymes in the cell-bound form. The second possibility is that Type I and Type II enzymes originate from the same protein in the cell. The solution of this problem requires the purification of both types and the determination of their amino acid sequences.

Recently, Soda *et al.*[22] purified, chrystallized, and characterized a kind of amino acylase from *Bacillus stearothermophilus*. Their enzyme contains 2 g atoms of zinc per mole of protein, however polymyxin acylase (Types I and II) contains calcium, and its substrate specificity is far from that of their enzyme. Murao *et al.*[23] reported some papers concerning urethane-hydrolyzing enzymes from *Lactobacillus fermenti*, but their enzymes did not act on Z-peptides. Polymyxin acylase (Type II) removed the Z group of some Z-peptides. We believe that both types of polymyxin acylase will have many practical applications in peptide chemistry; two obvious examples are in applying the Edman degradation to naturally occurring *N*-acyl peptides, and in preparing analogs of *N*-acyl peptides.

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